Immunological Effects of Cytokines and Anti-allergic Traditional Chinese Medicine on Human (HMC-1) Mast Cells

by

Tsang Chi Man

A Thesis Submitted in Partial Fulfillment
of the Requirements for the degree of
Master of Philosophy in
Chinese Medicine


香港中文大學
圖書館系統
18 NOV 2006
UNIVERSITY
LIBRARY SYSTEM
THE CHINESE UNIVERSITY OF HONG KONG

Acknowledgements

Special thanks should be given to Professor CWK Lam, the Chairman of the Department of Chemical Pathology, the Chinese University of Hong Kong, for his supervision and advice over my project. His enthusiastic working manner and willingness in helping students have motivated me to seek for different challenges and better achievement in my life. I have also benefited a lot from his sharing and guidance throughout my MPhil project.

I would also like to deliver my sincere gratitude to my co-supervisor, Professor CK Wong, Associate Professor of the Department of Chemical Pathology, the Chinese University of Hong Kong, for his valuable advice, fruitful discussion and detailed instructions on all my research works and publications. His continuous teaching and guidance have greatly sharpened my knowledge in the scientific field. I would also like to convey my deepest appreciation in his prompt help in solving any obstacles met in my research.

Thanks must also be given to Dr WK Ip for his technical assistance and guidance on my experiments. I will always remember his selfless teaching on the incomers and endless support to his colleagues. I also want to say thanks to TY Law, for her friendship and heartfelt care that helped me to go through some difficult moment. My gratitude also goes to all my colleagues of the Department of Chemical Pathology, for providing a working environment that is always filled with encouragements, laughter and joy.

Last but not least, I have to express my thanks to my family and HY Law, for their boundless love and care that enable me to cope with any ups and downs in life and provide me a forever shelter to take rest.

The work described in this thesis was substantially supported by a grant from the University Grants Committee of the Hong Kong Special Administrative Region, China under the Area of Excellence project "Chinese Medicine Research and Further Development"(Project No. AoE/B-10/01) coordinated by the Institute of Chinese Medicine of The Chinese University of Hong Kong

ABBREVIATIONS

AP-1	Activator protein-1
APC	Antigen presenting cells
APS	Ammonium persulfate
ATF-2	Activating transcription factor-2
BAL	Bronchoalveolar lavage
BLC	B lymphocyte chemoattractant
BSA	Bovine serum albumin
CBA	Cytometric beads array
CBP	CREB binding protein
CCR	CC chemokine receptor
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CREB	Cyclic AMP response element binding protein
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
dNTP	2'-Deoxyribonucleoside 5'-triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EGTA	Ethylene glycol-bis(β-aminoethyl Ether)-N, N, N', N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMAP	Endothelial-monocyte activating polypeptide
ERK	Extracellular signal-regulated protein kinase
Et Br	Ethidium bromide
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMC-1	Human leukemic mast cell line-1
ICAM-1	Intercellular cell adhesion molecule-1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium

IP-10	Interferon-inducible protein-10
IκB	Inhibitor kappa B
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
LFA-1	Leukocyte function-associated antigen-1
LT C4	Leukotriene C4
mAB	Monoclonal antibody
Mac-1	Macrophage antigen-1
MACS	Magnetic cell sorting system
MAPK	Mitogen-activated protein kinase
MAPKK or MEK	MAPK kinase or MAP/ERK kinase
MAPKKK or MEKK	MAPKK kinase or MEK kinase
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
MPIF	Macrophage procoagulant inducing factor
MTT	Thiazolyl blue tetrazolium bromide
NF-κB	Nuclear factor-κB
NK	Natural killer
PAF	Platelet activation factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered-saline
PBMC	Peripheral blood mononuclear cells
PKC	Protein kinase C
PVDF	Polyvinylidene difluoride
RANTES	Regulated upon activation normal T-cell expressed and secreted
RBL	Rat basophilic leukemia
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
SDF	Stromal cell-derived factor
SDS	Sodium dodecyl sulfate
SLE	Systemic lupus erythematosus
STAT	Signal transducers and activators of transcription
TAE	Tris-acetate buffer

TARC	Thymus and activation-regulated chemokine
TBP	TATA-binding protein
TBST	Tris-buffered saline Tween 20
TCM	Traditional Chinese Medicine
TEMED	N, N, N', N'-tetra-methylethylenediamine
TGF	Transforming growth factor
Th	T helper cells
TNF	Tumor necrosis factor
UV	Ultraviolet
VCAM	Vascular-cell adhesion molecule
VLA-4	Very late activated antigen-4

Abstract

Mast cells play pivotal roles in immunoglobulin (Ig) E-mediated airway inflammation and other mast cell-mediated inflammation by interacting and recruiting inflammatory cells after activated by cytokines in the inflamed tissues.

In the present study, we first investigated the intracellular signaling mechanisms regulating the induction of chemokines and adhesion molecules from human mast cell line-1 (HMC-1) under the effects of different cytokines. Based on the findings, we then assessed the *in vitro* anti-allergic activities of a Wheeze-relief formula composed of 5 herbs of traditional Chinese medicine (TCM) on HMC-1 cells.

To generate a profile of chemokine and adhesion molecule expression under the effects of cytokines, we assayed the expression of interleukin (IL)-8, monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T-cell expressed and secreted (RANTES), I-309, macrophage inflammatory protein (MIP)-1β, interferon-γ-inducible protein-10 (IP-10), intercellular adhesion molecule (ICAM)-1 and ICAM-3 of the HMC-1 cells after treating with stem cell factor (SCF), tumor necrosis factor (TNF)-α, IL-13, IL-18 and IL-25. IL-8, MCP-1, RANTES and IP-10 were quantitated by flow cytometry based cytometric beads array (CBA). ELISA was used to measure the release of I-309 and MIP-1β. Cell surface expressed ICAM-1 and ICAM-3 were assayed by flow cytometry. Gene expression levels were also studied using reverse transcription-polymerase chain reaction (RT-PCR).

Results showed that SCF and TNF-α could induce the release of IL-8, MCP-1, RANTES and I-309. MIP-1β and IP-10 were upregulated by SCF and TNF-α respectively. IL-13, IL-18 and IL-25 had no effect on all the chemokines. Expression of ICAM-1 could be induced by SCF, TNF-α and IL-13 but not IL-18 and IL-25. It was also shown that combined treatment of (SCF + IL-13) and (SCF + TNF-α) had an

additive and synergistic effect on the ICAM-1 expression respectively. All cytokines had no effect on the expression of ICAM-3.

Since SCF and TNF-α showed most potent effects on IL-8, MCP-1, RANTES, I-309, MIP-1β, IP-10 and ICAM-1, we then focused on their effects in activating intracellular signal transduction pathways of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB. Activation of these pathways was quantitatively assessed by ELISA. Selective inhibitors of the pathways including PD98059 for ERK pathway, SB203580 for p38 MAPK pathway and BAY117082 for NF-κB pathway, were also used to confirm the regulatory mechanisms for the effects of SCF and TNF-α.

We found that the SCF-induced upregulation of IL-8, MCP-1, RANTES, I-309. MIP-1β and ICAM-1 were mediated through ERK pathway. The TNF-α-induced upregulation of IL-8, MCP-1, I-309 and IP-10 were mediated by p38 MAPK pathway. Besides, NF-κB pathway was responsible to conduct the TNF-α-induced IP-10, RANTES and ICAM-1. The results also suggested that SCF-induced activation of ERK could enhance the TNF-α-induced activation of NF-κB pathway, thereby giving the synergistic upregulation of ICAM-1 expression.

To investigate the *in vitro* anti-allergic activities of the Wheeze-relief formula, we studied the effects of the five herbal components, including *Cordyceps sinensis* (冬蟲夏草), *Bulbus Fritillariae cirrhosae* (川貝), *Radix Stemonae* (百部), *Radix astragali* (黃耆) and *Radix scutellariae* (黃芩) on the SCF-induced release of I-309 and MCP-1 from the HMC-1. It was because I-309 and MCP-1 can specifically attract the T helper (Th)2-cells for bringing out allergic responses. However, the five herbs could not show a specific downregulation on the release of these 2 chemokines.

In conclusion, we have elucidated the different intracellular signaling pathways regulating the chemokine and adhesion molecule expression of SCF and TNF-α

activated mast cells. It provides important information for generating biological and pharmacological implications on treating mast cell-mediated diseases. Besides, we have also shown that these studies may help to develop parameters to investigate the therapeutic mechanisms of TCM.

撮要

炎症組織中的肥大細胞，經過細胞因子活化後，可與其他炎性細胞發生相互作用，因此其在免疫球蛋白 E(IgE)介導的氣道炎症中具有重要作用。

在本研究中，我們首先探討了不同細胞因子作用下，人肥大細胞絲 (HMC)-1 產生趨化因子及黏附分子的細胞內信號傳導機制。在此研究結果基礎上，通過合適的研究平台，系統研究了由五種中藥所合成的平息氣喘劑的體外抗過敏活性。

爲了探明肥大細胞經細胞因子作用後，趨化因子及細胞間黏附分子的表達情況，我們檢測了經幹细胞因子 (stem cell factor, SCF), 腫瘤壞死因子 (tumor necrosis factor,TNF)-α, IL-13, IL-18 和 IL-25 刺激後，白介素 (interleukin, IL)-8, 單核细胞趨化蛋白 (monocyte chemoattractant protein, MCP)-1, 激活時可調節的正常 T 细胞表達和分泌因子 (regulated upon activation normal T-cell expressed and secreted , RANTES), I-309, 巨噬细胞炎症蛋白(macrophage inflammatory protein , MIP)-1β, γ干擾素誘生蛋白 (interferon-γ-inducible protein, IP)-10, 細胞間黏附分子(intercellular adhesion molecule, ICAM)-1 和 ICAM-3 的表達。IL-8, MCP-1, RANTES 和 IP-10 用流式蛋白分析系統 (cytometric beads array, CBA)來定量。用酶聯免疫法 (ELISA) 測定 I-309 和 MIP-1β。細胞表面 ICAM-1 和 ICAM-3 用流式細胞儀檢測。而基因表達則用逆轉錄-聚合酶鏈反應 (reverse transcription- polymerase-chain-reaction, RT-PCR) 分析。

結果顯示，SCF 和 TNF-α 可以誘導 HMC-1 細胞釋放 IL-8, MCP-1, RANTES 和I-309。MIP-1β and IP-10 可分別被 SCF 和 TNF-α 上調。IL-13, IL-18 和 IL-25 對所有趨化因子釋放無明顯影響。ICAM-1 的表達可被 SCF, TNF-α 和 IL-13 誘導，但不能被 IL-18 和 IL-25 所誘導。SCF 及 IL-13，和 SCF 及 TNF-α 聯合刺激對 ICAM-1 的表達有綜合及協同作用。上述所有細胞因子對 ICAM-3 表達都無明顯作用。

由於 SCF 和 TNF-α 對 IL-8, MCP-1, RANTES, I-309, MIP-1β, IP-10 和 ICAM-1 在 HMC-1 細胞表達都有顯著誘導作用，所以我們重點研究了它們對細胞內信號傳導通路中的细胞外信號調節激酶(extracellular signal-regulated kinase,

ERK)，p38 絲裂原活化蛋白激酶 (mitogen-activated protein kinase, MAPK) 和核因子(nuclear factor, NF)-κB 的影響。這些酶活性用酶聯免疫法所分析。運用信號傳導道路中酶的選擇性抑制劑：抑制 ERK 的 PD98059, 抑制 p38 MAPK 的 SB203580 和抑制 NF-κB 的 BAY117082，進一步確認 SCF 和 TNF-α 活化 HMC-1 細胞的信號傳導途徑。

研究結果發現，經 SCF 誘導的 IL-8, MCP-1, RANTES, I-309, MIP-1β 和 ICAM-1 表達是經由 ERK 途徑。TNF-α 誘導的 IL-8, MCP-1, I-309 和 IP-10 的釋放是通過 p38 MAPK 途徑。而 TNF-α 誘導的 IP-10, RANTES 和 ICAM-1 的表達則是通過 NF-κB 途徑。結果還顯示經 SCF 誘導的 ERK 活性可提升 TNF-α 誘導的 NF-κB 路徑的活性，並對 ICAM-1 表達具有協同作用。

爲了探討中藥平喘單方體外抗敏活性，我們研究了五種中藥： 冬蟲夏草 (*Cordyceps sinensis*), 川貝 (*Bulbus Fritillariae cirrhosae*), 百部 (*Radix Stemonae*), 黃耆 (*Radix astragali*) and 黃芩 (*Radix scutellariae*) 對 SCF 活化的 HMC-1 細胞釋放 I-309 和 MCP-1 的影響。因爲 I-309 和 MCP-1 可以選擇性吸引 II 型輔助型 T 細胞 (T helper cells type 2)從而引發過敏反應。但上述五種中藥對活化後 HMC-1 細胞釋放 I-309 和 MCP-1 均無顯著影響。

總之，通過上述系統研究，我們已經探明 SCF 和 TNF-α 活化肥大細胞表達趨化因子及黏附分子的不同細胞內信號傳導通路。本研究結果爲肥大細胞介導疾病的治療提供了重要的生物及藥理信息，而且也可以爲中草藥藥理學機制提供理論基礎。

Publications

Refereed Publications:

Tsang CM, Wong CK, Ip WK, Lam CW. 2005. Synergistic effect of SCF and TNF-{alpha} on the up-regulation of cell-surface expression of ICAM-1 on human leukemic mast cell line (HMC)-1 cells. J Leukoc Biol. 78: 239-247

Wong CK, **Tsang CM,** Ip WK, Lam CW. 2005. Molecular mechanisms for the release of chemokines from human mast cells activated by SCF and TNF-α: roles of ERK, p38 MAPK and NF-κB. Allergy [in press]

Abstracts of Conference:

Tsang CM, Wong CK, Ip WK, Lam CW. Synergistic effect of SCF and TNF-{alpha} on the up-regulation of cell-surface expression of ICAM-1 on human leukemic mast cell line (HMC)-1 cells. 34th Annual Meeting of Japanese Society for Immunology, 2004. Conference supplement, page 112. [Travel Award]

Tsang CM, Wong CK, Ip WK, Lam CW. Intracellular signal transduction regulating cytokine-mediated cell surface expression of ICAM-1 on human HMC-1 mast cells. 9th Annual General Meeting and Scientific Meeting, Hong Kong Society of Flow Cytometry, 2004. [Oral presentation]

Wong CK, **Tsang CM**, Ip WK, Lam CW. Intracellular signaling mechanisms of cytokine-regulated cell surface expression of ICAM-1 on human HMC-1 mast cells Proceeding from the 10th Asian Pacific Congress of Clinical Biochemistry and 42nd annual Scientific Conference of the Australasian Association of Clinical Biochemists, 2004. The Clinical Biochemist Reviews, supplement, page S63. [Poster abstract]

Table of contents

Chapter 4 Discussion

Chapter 1
Introduction

1.1 Human mast cells and their pathological roles in inflammation

1.1.1 Morphology of mast cells

Mast cells are leukocytes ranging from 5 to 30 μm in diameter. The shape of the mast cells vary dependent on tissue location and has been described as polyhedral, fusiform, ovoid, and rectangular with a round or oval nuclei [He et al, 2004]. The most characteristic feature of mast cells is their cytoplasmic granules which vary in size from 0.2 to 0.5 μm in diameter that can occupy up to 40% of the volumn of mast cells [Bischoff et al, 2002].

1.1.2 Mediators of mast cells

Numerous mediators are stored in metachromatic granules and can be released at the time of stimulation to bring out the biological functions. The mediators are generally divided into three subclasses: (1) preformed mediators including histamine, tryptase, chymase and proteoglycans; (2) newly generated mediators including eicosanoids, leukotrienes, prostaglandins and platelet activating factor [Kawakami et al, 2002]; and (3) an array of cytokines and chemokines, including interleukin (IL)-4, IL-5, IL-6, tumor necrosis factor (TNF)-α, IL-1β, IL-8, IL-13, IL-18, IL-25, granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), macrophage chemotactic peptide (MCP)-1, 2, 3, regulated on activation of normal T cell-expressed and secreted protein (RANTES) and eotaxin [He et al, 2004].

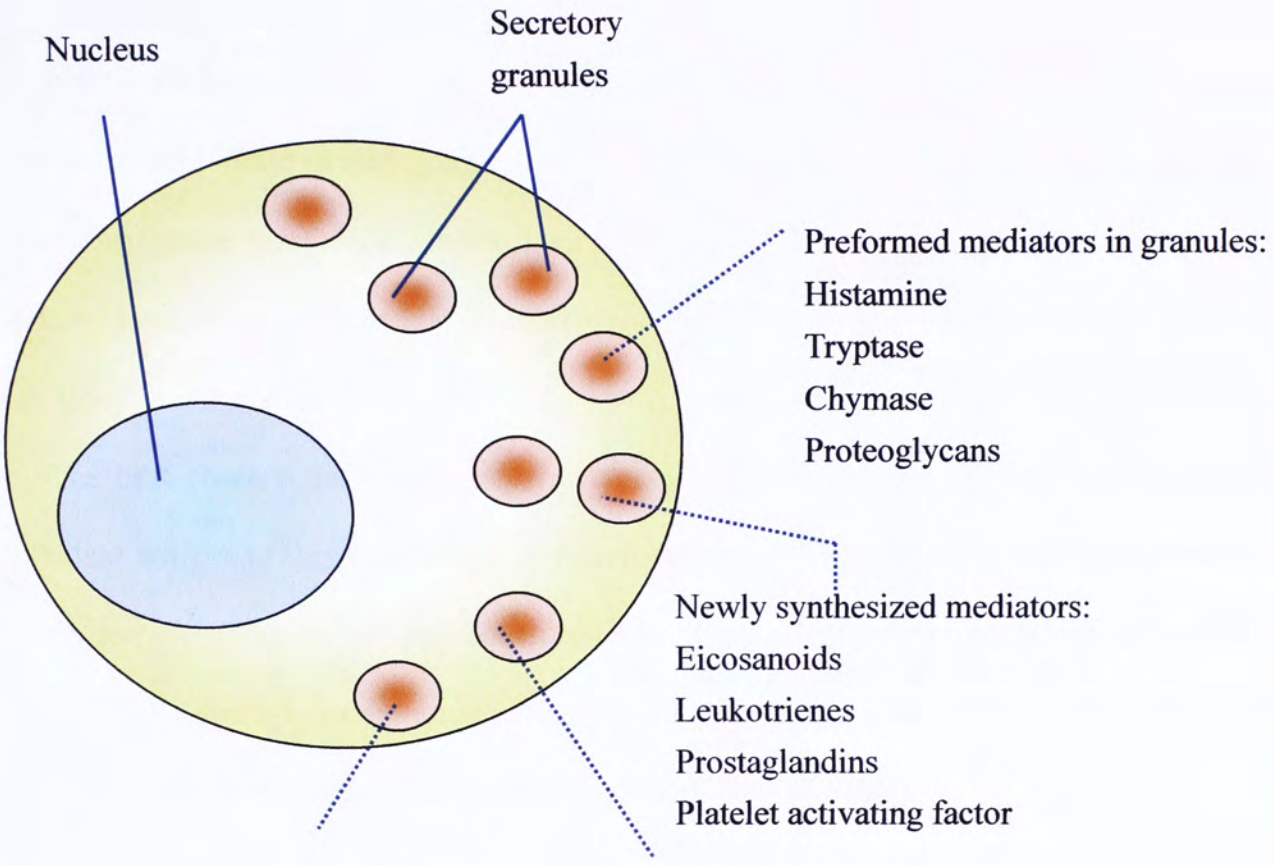


Figure 1.1 Basic structures of human mast cell and its mediators

1.1.3 Migration and activation

Mast cells arise from multipotent CD34+ precursors in the bone marrow and circulate in the peripheral blood as non-granular, monocytic appearing cells. After migrating into tissues, these immature mast cells assume their typical granular morphology and become CD34-/ c-kit+/ FcεRII(low affinity IgE receptor)- and FcεRI(high affinity IgE receptor)+ [Rottem et al, 1994].

The best characterized stimulus of mast cells is bridging of FceRI receptors by specific divalent antigens. This event leads to fusion of mast cells secretory granule membrane with the plasma membrane and subsequent release of granule contents. Mast cell activation can also occur through exogenous agents, such as antigens and opiates; and endogenous molecules, such as cytokines and autoantibodies [He et al, 2004].

1.1.4 Pathological roles of mast cells

Mast cells are historically known to be the central effector cells in type 1 hypersensitivity such as allergic bronchial asthma, atopic rhinitis and urticaria [Pawankar et al, 2003]. It is because mast cells have been well demonstrated for their IgE-mediated release of mediators in the immediate phase reaction of allergic diseases [Oliveira et al, 2001]. The mediators released upon allergen stimulation can lead to the symptoms of allergy by increasing vasopermeability, contracting smooth muscle and increasing mucus production. Mast cells have also been shown to play their crucial roles in late phase allergic response and chronic allergic inflammation by attracting T lymphocytes, neutrophils, basophils and eosinophils and promoting the T helper cell type 2 (Th2)-mediated inflammations [Marone et al, 2002].

Until the last two decades, human mast cells have also been recognized in producing a wide spectrum of cytokines and chemokines. It consequently leads to the exploration of the

both physiologic and pathologic roles of mast cell-derived cytokines and chemokines in diseases [He et al, 2004]. It is then revealed that mast cells are not only crucial for allergic responses, but also the non-allergic inflammations, innate and adaptive immunity [Woolley, 2003; Pawankar et al, 2003]. There is now strong evidence showing the involvement of mast cells in a number of non-allergic diseases. For example, markedly increased number of mast cells was observed in the mucosa of the ileum and colon of patients with inflammatory bowel disease, which was accompanied by degraunulation of the content of mast cells. Pathogenesis of other non-allergic diseases including chronic obstructive pulmonary disease (COPD), Crohn's disease, ulcerative colitis, multiple sclerosis and rheumatoid arthritis, etc also shows mast cell is the key involved cell type. Table 1.1 shows the evidence of mast cells involvement in non-allergic diseases as adopted from He et al. 2004.

Table 1.1. Mast cell involvement in non-allergic diseases

Disease	evidence
chronic obstructive pulmonary disease (COPD)	mast cell hyperplasia in eipithelia and bronchial glands, tryptase and histamine release in BALF
Crohn's disease	mast cell hyperplasia and degranulation
ulcerative colitis	mast cell hyperplasia and degranulation
multiple sclerosis	mast cell hyperplasia
rheumatoid arthritis	mast cell degranulation
cystic fibrosis	mast cell hyperplasia and degranulation in lung

Recently, mast cells are also shown to be involved in innate and adaptive immunity. Mast cells show their role in innate immunity by recognizing specific molecular patterns from microbials through binding with Toll-like receptors and trigger inflammatory responses [Jeffrey et al, 2003]. C3a, one component of complement system that is essentially linked with innate immunity against bacteria and other pathogens, can also induce the release of chemokines form mast cells [Venkatesha et al, 2005]. Mast cells have also been shown to

migrate from a site of antigen encounter, via afferent lymphatics to the draining lymph nodes, wherein they participate in induction of a primary immune response [Wang et al, 1998].

Together, mast cells are important effector cells in allergic responses, chronic allergic inflammation, non-allergic diseases and innate and primary immunity by releasing allergic mediators, cytokines and chemokines for autocrine and paracrine activation of the inflamed tissue [Walsh et al,1991; Oliveira et al, 2001].

1.1.5 Human mast cell-1 (HMC-1)

Mast cells are not normally found in human blood circulation and culture of mast cells from peripheral blood or inflamed tissues is very difficult and time consuming [Kimata et al, 2000]. Therefore, investigation of human mast cells has been mostly relied on a human leukemic mast cell line (HMC-1) [Butterfield et al, 1998] as the *in vitro* mast cell model. Figure 1.2 shows the microscopic slide of HMC-1 cells using cytospin. HMC-1 is the mast cell line established from the peripheral blood of a patient with mast cell leukemia and retains the characteristic of immature mast cell expressing a low level of high affinity IgE (FcεRI) receptor. Detail phenotypic characterization has been done and shown to possess many characteristics of human mast cells including the expression of c-kit (SCF receptors) and the production of inflammatory mediators such as histamine, tryptase, leukotrienes and prostaglandins [Welker et al, 2001]. Table 1.2 shows the presence of mast cells markers in HMC-1 cells. HMC-1 is the only growth factor independent human mast cell line, and is well accepted to serve as an *in vitro* model to study basic mast cell functions and signal transduction [Kempna et al, 2004; Fitzgerald et al, 2004].

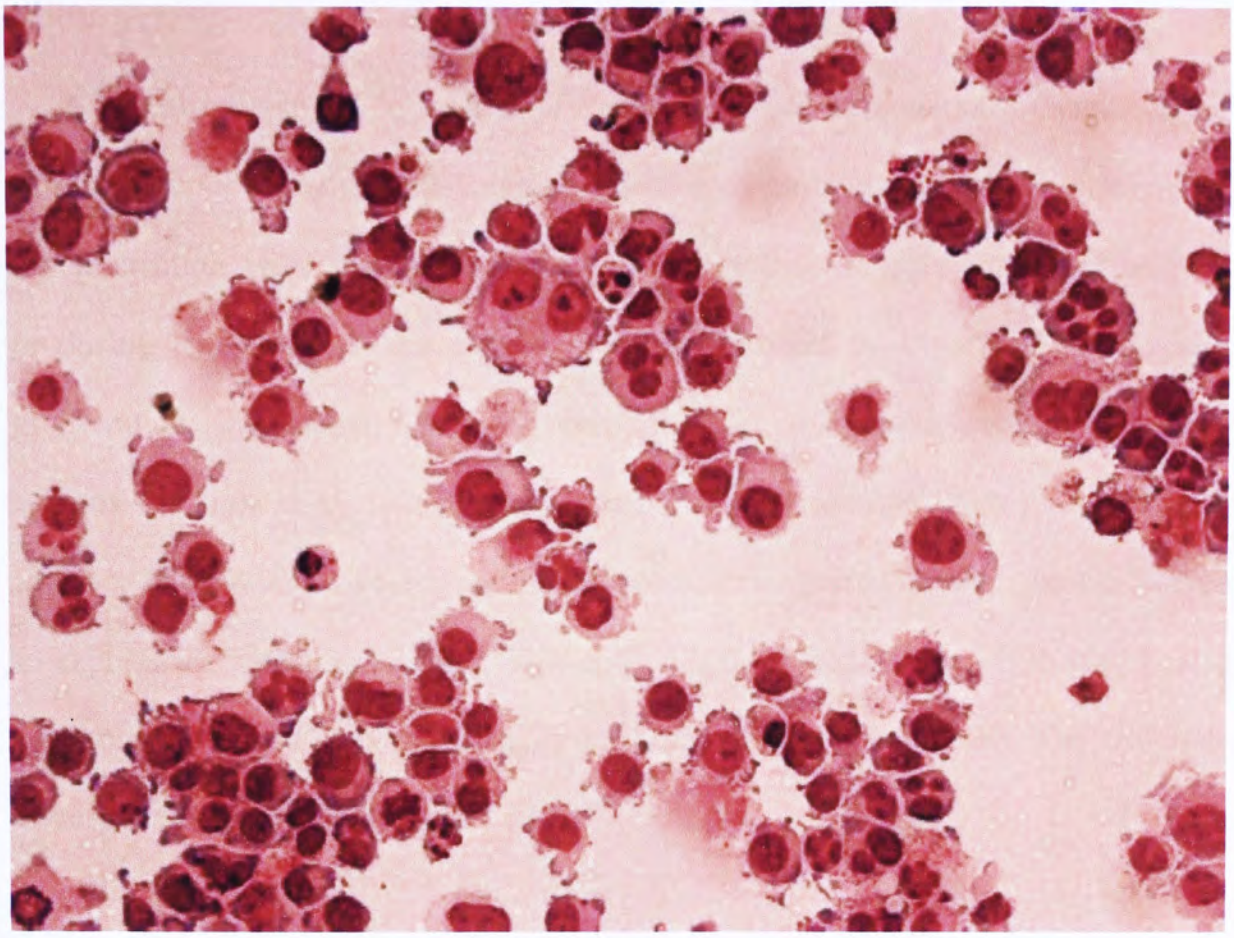


Figure 1.2 Photograph of HMC-1 cells under light microscope. The cells were centrifuged at 300 rpm for 3 min onto a microscopic slide using cytospin and then air-dried following by staining with Hemacolor rapid staining set.

Table 1.2 The presence of mast cell markers on HMC-1 cells (adopted from Grabbe et al, 1998)

Parameter measured	**HMC-1 cells**
Tryptase (ng/10^6 cells)	150 ± 55
FcεRIα (high affinity IgE receptor, % of positive cells)	7.1 ± 3.2
Histamine (μg/10^6 cells)	0.29 ± 0.28
c-kit (SCF receptor, % of positive cells)	79.0 ± 9.3

1.2 Cytokines as stimulator of mast cells in inflammation

As mentioned in 1.1.3, mast cells can be activated by cytokines. Cytokines are small extracellular signaling proteins exerting effects by paracrine and autocrine manner, through the interaction with specific receptors on the surface of adjacent target cells or cells producing themselves. Some cellular functions initiated by cytokines include activation, proliferation, chemotaxis, release of other cytokines or mediators, cell differentiation, and apoptosis [Chung et al, 1999]. When inflammation occurs, cytokines produced by various cells act on each other to bring out the inflammatory responses. Human mast cells are shown to express the receptors for SCF, TNF-α, IL-13, IL-18 and IL-25, which play key roles in activating mast cells or conducting inflammatory responses after the stimulation of corresponding ligands [Wedi et al, 1996].

1.2.1 SCF

SCF is a stromal cell-derived cytokine synthesized by fibroblasts in the peripheral tissue, eosinophils, airway smooth muscle cells, endothelial cells, and also mast cells themselves [Oliveria et al, 2003]. The receptor for SCF is the c-kit. The receptor has been associated with the activation of PI3-kinase, Akt and ERK pathways [Bondzi et al, 2000].

SCF is well known as a cytokine capable of regulating mast cell number and functions under physiological conditions. It is a growth factor that can induce differentiation and promote survival of mast cells [Oliveria et al, 2003]. At concentrations approximately 10-100 fold lower than those eliciting cell proliferation, SCF promotes the synthesis of histamines and leukotriene C4 in mast cells [Oliveria et al, 2001]. Mast cells predominantly produce pro-inflammatory cytokines including IL-1, IL-6, IL-8, IL-16 and IL-18 in the presence of SCF [Lorentz et al, 2001]. It is also a potent chemotactic factor for mast cells by inducing

rearrangement of the actin filaments of the cytoskeleton [Gebhardt et al, 2005].

1.2.2 TNF-α

TNF-α is produced from many cells including macrophages, T-cells, eosinophils, epithelial cells and also mast cells themselves, but the principal source is macrophages. TNF-α interacts through two related receptors, TNF-R1 and TNF-R2. The two receptors have been identified to activate nuclear factor (NF)-κB, AP-1 and mitogen activated protein kinase (MAPK) intracellular signaling pathways [Chung et al, 1999].

TNF-α is an important mediator in many cytokine-dependent inflammatory events. It has been demonstrated to be capable of stimulating airway epithelial cells to produce different cytokines including RANTES, IL-8, and GM-CSF [Berkman et al, 1995; Cromwell *et al,* 1992] in allergic asthma. It is known to up-regulate adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in facilitating inflammatory cell migration [Thomas, 2001]. TNF-α can also act as a chemotactic cytokine for eosinophils and neutrophils [Ming et al, 1987].

1.2.3 IL-13

IL-13 is expressed in activated CD4+ T-cells, CD8+ T-cells and mast cells. The receptor of IL-13 is IL-13 receptor that is expressed on macrophages, B-cells, eosinophils and mast cells [Wills-Karp, 2004].

IL-13 is a potent stimulator of inflammation and tissue remodeling that plays a key role in the pathogenesis of a wide variety of human disorders. IL-13 is a key inducer of several Th2 cytokine-dependent pathologies. It regulates inflammation, mucus production, tissue remodeling, and fibrosis [Mentink-Kane et al, 2004]. IL-13 induces human monocyte

differentiation, enhances survival time in culture, and also induces B-cell differentiation, proliferation and isotype switching [Jelinek et al, 2000]. IL-13 down-modulates macrophage activity, reducing the production of proinflammatory cytokines (IL-1, IL-6, IL-8, IL-10, IL-12) and chemokines in response to IFN-γ or bacterial lipopolysaccharides (LPS) [Wills-Karp, 2004]. IL-13 also decreases the production of nitric oxide by activated macrophages, leading to a decrease in parasiticidal activity.

1.2.4 IL-18

IL-18 is expressed by many cell types including monocytes and macrophages, T-cells, B-cells, mast cells, dendritic cells, intestinal epithelial cells [Dinarello, 1999; Nakanishi et al, 2001] and its receptor is IL-18 receptor [Xu et al, 1998].

IL-18 is crucial in promoting Th1 cytokine responses, primarily by its ability to induce interferon (IFN)-γ production in T cells, B-cells and natural killer (NK) cells. IL-18 can also up-regulate the cytotoxic actions of NK and CD8+ T cells by enhancing the expression of Fas ligand on their surfaces [Hoshino et al, 1999; Dao et al, 1996].

Though the importance of IL-18 in enhancing the Th1 responses, several reports have shown its role in Th2 responses. A murine model of allergic asthma has illustrated that IL-18 can augment allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia [Wild et al, 2000]. Besides, the mRNA level of IL-18 was significantly increased in nasal mucosa after nasal allergen provocation in patients with allergic rhinitis [KleinJan et al, 1999].

1.2.5 IL-25

IL-25 is secreted by bone marrow stromal cells, CD4+activated memory T cells and mast cells. Its receptor is IL-17 receptor homology 1 (Rh1) [Kawaguchi et al, 2004].

IL-25 is a novel Th2 proinflammatory cytokine that can provoke allergic inflammation in atopic disease and late phase allergic reactions. Intranasal administration IL-25 induces eosinophilia in the bronchoalveolar lavage and lung tissue and increases the production of IL-4, IL-13 and eotaxin mRNA in the lung [Fort et al, 2001]. It has been shown that IL-25 can induce chemokines and IL-6 release from eosinophils (Wong et al, 2005). Mice develop epithelial cell hyperplasia and airway hyperreactivity after intranasal administration of IL-25 while knock out mice lacking IL-25 receptor do not develop eosinophilia in response to IL-25 [Hurst et al, 2002].

1.3 Interaction of mast cells with inflammatory cells through adhesion molecules and chemokines

Mast cells have been shown to interact with other inflammatory cells so as to mediate allergic and non-allegic inflammation. One of the earliest interaction with other cells is to bind with endothelial cells through cell surface expressed adhesion molecules, so that the mast cells can leave the blood circulation and enter the inflammatory sites [Pawankar et al, 2003]. In fact, all the mast cell-mediated inflammations are characterized by an accumulation of mast cells in the inflamed tissue [Bischoff et al, 2002]. Within the inflammatory site, mast cells produce an array of mediators, cytokines or chemokines to activate or recruit other inflammatory cells [Frossi et al, 2004].

1.3.1 Adhesion molecules on mast cells

ICAM-1 and ICAM-3 have been shown to be associated with recruitment of mast cells to inflamed tissues. Apart from their well recognized roles in leukocyte trafficking and cell-extracellular matrix adhesion, they can also transduce messages after binding with their ligands leading to degranulation or activation of the cells.

(1) ICAM-1

ICAM-1 is a 80 to 114 kDa calcium-independent transmembrane glycoprotein containing five Ig-like domains [Staunton et al, 1988]. Its ligands are lymphocyte function associated antigen-1 (LFA-1), macrophage antigen-1 (Mac-1), fibrinogen, hyaluronan and CD43 [van de Stolpe et al, 1996].

ICAM-1 can be up-regulated in response to a variety of inflammatory mediators, including

proinflammatory cytokines, hormones, cellular stresses, and virus infection [Roebuck KA et al, 1999]. It is an important early marker of immune activation and involved in bronchial asthma, allergic rhinitis [Grzelewska-Rzymowska et al, 2004] and inflammatory bowel disease [Papa A et al, 2004]. Its interactions with the beta2 integrins LFA-1 and MAC-1 on the surface of leukocytes are important for their transendothelial migration to sites of inflammation and it also functions as costimulatory molecules for T cell activation [Roebuck KA et al, 1999]. It is also suggested that activated T cells mediate mast cell degranulation via ICAM-1-LFA-1 interaction [Inamura et al, 1998].

(2) ICAM-3

Similar to ICAM-1, ICAM-3 also contains five Ig domains. The ligand is LFA-1 but not Mac-1 [Fawcett et al, 1992]. It is generally expressed at high levels on resting mast cells. ICAM-3 has been proposed to be an adhesion molecule that is particularly important in the initiation of immune responses, because of its higher level of constitutive expression on lymphocytes compared with ICAM-1 and ICAM-2 [de Fougerolles et al, 1994]. It has also been reported that engagement of ICAM-3 on monocytes and polymorphonuclear cells by specific immobilized mAbs induces secretion of chemokines and cellular spreading [Kessel et al, 1998]. Its ligation can lead to polymorphonuclear cell aggregation and adhesion to immobilized ligands [Feldhaus et al, 1998].

1.3.2 Chemokines released by mast cells

Chemokines are a group of small (8 to 12 kDa) molecules able to induce chemotaxis in a variety of cells including neutrophils, monocytes, lymphocytes, eosinophils, fibroblasts, and keratinocytes. They are crucial during inflammatory responses for a timely recruitment of

specific leukocyte subpopulations [Moser et al, 2004]. Along with the accelerated rate of investigating the chemokines has come the realization that they not only control cell migration, but are also involved in a number of pathological processes through cell activation [Bisset et al, 2005].

Most chemokines have four characteristic N-terminal cysteines, and dependent on the motif displayed by the first two cysteines, they are classified into into four major groups given the preferred names CC, CXC, C and CX_3C [Luster, 1998].

IL-8, interferon-inducible protein-10 (IP-10), RANTES, MCP-1, I-309 and macrophage inflammatory protein (MIP)-1β have been shown to be released by mast cells and take different roles during inflammation [Selvan et al, 1994].

(1) IL-8

IL-8 is a proinflammatory CXC chemokine binding to CXC chemokine receptor-1 and 2 (CXCR1 and CXCR2) which are mainly expressed on neutrophils. Significant correlations between plasma IL-8 concentration and neutrophil infiltration in acute inflammation has been reported [Huber et al, 1991].

Besides, IL-8 can regulate various biological functions of neutrophils including chemotaxis, degranulation, superoxide production, and expression of adhesion molecules. In eosinophil, IL-8 can induce shape change and release of eosinophil peroxidase from eosinophils of patients with hypereosinophilic syndrome [Kernen et al, 1991].

(2) MCP-1

MCP-1 is a highly inducible inflammatory CC chemokine. The effect of MCP-1 is exerted through receptor, CCR2, which is expressed on monocytes, T-cells, NK-cells, and basophils

but not eosinophils [Rollins, 2001].

Apart from the chemotactic properties, it enhances the proliferation response of naive T-cells to anti-CD3 and B7-1-induced costimulation [Taub et al, 1996], and has been reported to polarize naïve T-cells toward Th2 responses when challenged by antigen [Karpus et al, 1997]. As for monocytes, MCP-1 enhances adhesion by up-regulating several adhesion molecules including CD11b, CD11c and CD18 [Vaddi et al, 1994]. In addition, MCP-1 can mediate inflammatory response by stimulating IL-1 and IL-6 expression from monocytes and histamine release from basophils [Kuna *et al,* 1992].

(3) RANTES

RANTES is a CC chemokine binding to CCR1, CCR4 and CCR5 receptors which are expressed on T-cells, monocytes, eosinophils and basophils. Therefore, RANTES is chemotactic for these cell types and plays an active role in recruiting leukocytes into inflammatory sites [Zhang et al, 1994].

RANTES also activates eosinophils to release, for example, eosinophilic cationic protein making it often associating with diseases such as asthma and allergic rhinitis [Lampinen et al, 2004]. It also increases the adherence of monocytes to endothelial cells [von Hundelshausen et al, 2004]. RANTES activates human basophils to release histamines [Conti et al, 1997].

(4) IP-10

IP-10 is a CXC chemokine. Its effect is mediated through the CXCR3 receptor that is expressed on Th1 cells, monocytes and neutrophils. Therefore, IP-10 selectively chemoattracts Th1 cells, monocytes and neutrophils [Zeng et al, 2005]. Increased levels of IP-10 are found in psoriatic plaques characterized by the infiltration of neutrophils [Gottlieb

et al, 1988].

IP-10 also takes up roles other than chemotaxis by inhibiting cytokine-stimulated hematopoietic progenitor cell proliferation. Additionally, it is angiostatic and mitogenic for vascular smooth muscle cells [Yang et al, 2004]. It has also been suggested that IP-10 may play an important role in hypersensitivity reactions of the delayed type [Akahira-Azuma et al, 2004].

(5) I-309

I-309 is a CC chemokine that can act through the CCR8 receptor which is expressed on monocytes and Th2 cells [Haque et al, 2001]. It therefore chemoattracts these cell types and favors Th 2-associated allergic reactions [D'Ambrosio et al, 1998].

I-309 also exhibits an anti-apoptotic activity. The overexpression of this chemokine inhibited apoptosis in adult T-cell leukemia cells and can substantially contribute to their growth [Ruckes et al, 2001]. It can also protect thymic lymphomas against corticoid-induced apoptosis [Louahed et al, 2003].

(6) MIP-1β

MIP-1β is a CC chemokine. It exerts its effect through CCR1 and CCR5 [Rossi et al, 2000]. MIP-1ß preferentially attracts CD4+ T cells [Taub et al, 1993].

It is also involved in the cell activation of human granulocytes (neutrophils, eosinophils, and basophils) and appears to be involved in acute neutrophilic inflammation [Schall et al, 1993]. It can also augment the adhesion of CD8 + T-cells to the VCAM-1 and it does so by being present on the surface of endothelial cells complexed with endothelial proteoglycans [Tanaka et al, 1993].

1.4 Intracellular signaling pathways in mast cells

Recently, there have been increasing interests in the elucidation of the regulatory mechanisms of mast cell activation, differentiation, chemotaxis, adhesion, cytokine and chemokine productions and degranulation. One way to demonstrate the regulatory mechanisms is to study the activation of different signal transduction pathways under different stimulations. Signaling pathways direct the cellular functions by transducing the messages from the membrane receptors through a stream of signaling molecules, in which activated p38-mitogen-activated protein kinases (MAPK), extracellular-regulated protein kinase (ERK) and nuclear factor-kappa B (NF-κB) have been shown to regulate inflammatory responses [Wong et al, 2004].

1.4.1 The p38 MAPK pathway

p38 MAPK has been shown to be an important regulator of inflammation [Sundstrom et al, 2003] by regulating the cellular degranulation, chemotaxis, and adhesion molecules in allergic inflammation. Besides, its activation mediates inflammatory cell adhesion by inducing the expression of different adhesion molecules such as ICAMs and VCAMs [Johns et al, 2005]. The p38 MAPK is also an important mediator of the cellular response to environmental stress. It is activated by osmotic stress, UV irradiation, ionizing radiation, bacterial cell wall product LPS, and various proinflammatory cytokines including TNF-α and IL-1β (Raingeaud et al, 1995; Kyriakis & Avruch, 2001). Besides, it helps in regulating other physiological processes including cell cycle progression, differentiation and apoptosis.

Upon activation, p38 MAPK is subjected to dual phosphorylation at the Thr-Gly-Tyr motif. The phosphorylated p38 then activates a variety of transcription factor including activating transcription factor (ATF)-2, signal transducer and activators of transcription (Stat)-1,

Max/Myc complexes, MEF-2A/C, Elk-1 and cAMP responsive element binding protein (CREB). On the other hand, the upstream kinases acting on p38 include MAPK kinases (MKK) 3 and 6 which are in turn activated by MAPK/ERK kinases (MEKKs), MLKs and ASK1 [Roux et al, 2004]. Figure 1.3 shows the signaling pathway of p38 and the inhibitor SB 203580 that can inhibit the kinase activity of p38 MAPK.

1.4.2 The ERK pathway

ERK pathway has been well known for its involvement in cell proliferation, movement, and differentiation [Eblen et al, 2002, Hashimoto et al, 2000]. The ERK is shown to be activated by growth factor like IL-5 for the regulation of cell proliferation, transformation and differentiation [de Groot et al, 1998]. Recently, ERK activation is also found to be related to inflammations. Its inhibition can inhibit lung inflammation using a murine asthma model by suppressing the production of Th2 cytokines [Chialda et al, 2005]. Activation of ERK can also induce the release of chemokine IL-8 from neutrophils in inflammatory responses [Jo et al, 2004].

ERK functions downstream of the ras oncogene. Ras is activated in response to agonist stimulation and recruits Raf protein kinases to the plasma membrane [Leevers et al, 1994], where Raf undergoes activation by a mechanism that is incompletely understood. Raf proteins phosphorylate and activate ERK kinases (MEKs), which phosphorylate and activate the two ERK proteins, ERK1 and ERK2. MEKs can serve as cytoplasmic anchors for the ERKs through a direct binding interaction, holding the ERKs in the cytoplasm at times when the signaling pathway is inactive [Fukuda et al, 1990]. Stimulation of the pathway results in the phosphorylation of the ERKs and their dimerization and translocation to the nucleus and other sites of action, where they phosphorylate transcription factors e.g. Elk-l, Stat 1 and 4, c-myc and ER for bringing out biological responses [Eblen et al, 2002]. Figure 1.4 shows the

signaling pathway of ERK. PD98058 is the selective and cell permeable inhibitor of MAPK kinase (MEK) that acts by inhibiting the activation of ERK and subsequent phosphorylation of substrate.

1.4.3 The NF-κB Pathway

NF-κB is a ubiquitous transcription factor that appears to be of particular importance in cytokine-mediated inflammatory and immune responses. Activation of NF-κB can enhance the transcription of a variety of genes, including cytokines and growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins [Blackwell et al, 1997]. It is highly activated at inflammation tissues in various diseases including allergic asthma by enhancing the recruitment of inflammatory cells and production of pro-inflammatory cytokines [Ip et al, 2003]. Inhibition of NF-κB activity is effective at controlling inflammatory diseases in several animal models [Wong et al, 2003].

In unstimulated cells, NF-κB is localized to the cytoplasm because of binding to inhibitory protein, IκB [Baldwin, 1996]. When the cell is activated, specific IκB kinase phosphorylate IκB, leading to the rapid addition of ubiquitin and subsequent degradation in proteasome by the action of protease [Baldwin, 1996]. This allows NF-κB to be transported to the cell nucleus, where its dimers are free to bind to specific motifs in the promoter regions of various genes, and initiate transcription. Figure 1.5 shows the signaling pathway for NF-κB. BAY117082 can selectively and inreversibly inhibit the phosphorylation of IκB-α, resulting in a decreased expression of NF-κB.

1.4.4 Cross-talking of pathways

In 1.4.1, 1.4.2 and 1.4.3, p38 MAPK, ERK and NF-κB pathways were illustrated as

separate mechanisms starting from the membrane receptors, series of kinases and ultimately to transcriptional factors. However, signaling pathways are never discrete pathways but interact to from an integrated network by which precise and coordinated biological responses are given out.

A previous report has demonstrated that p38 MAPK is required for NF-κB-dependent expression of cytokines genes by the modulation of DNA binding of TATA–binding protein to the TATA box [Carter et al, 1999]. Inhibition of p38 MAPK can therefore potentially attenuate NF-κB-dependent transcription [Heinrich et al, 2000]. The two MAPK pathways, p38 and ERK, facilitate their cross-talking through scaffold proteins and adaptor molecules. Several MEK family members contain sites that can be phosphorylated by other kinases in p38 MAPK so that integration of signals can occur [Underwoon et al, 2000]. Moreover, Jiang et al [2002, 2004] demonstrated that ERK activity is required for persistent NF-κB activation and revealed a novel functional role for ERK as an important temporal regulator of NF-κB activation and NF-κB-dependent gene expression.

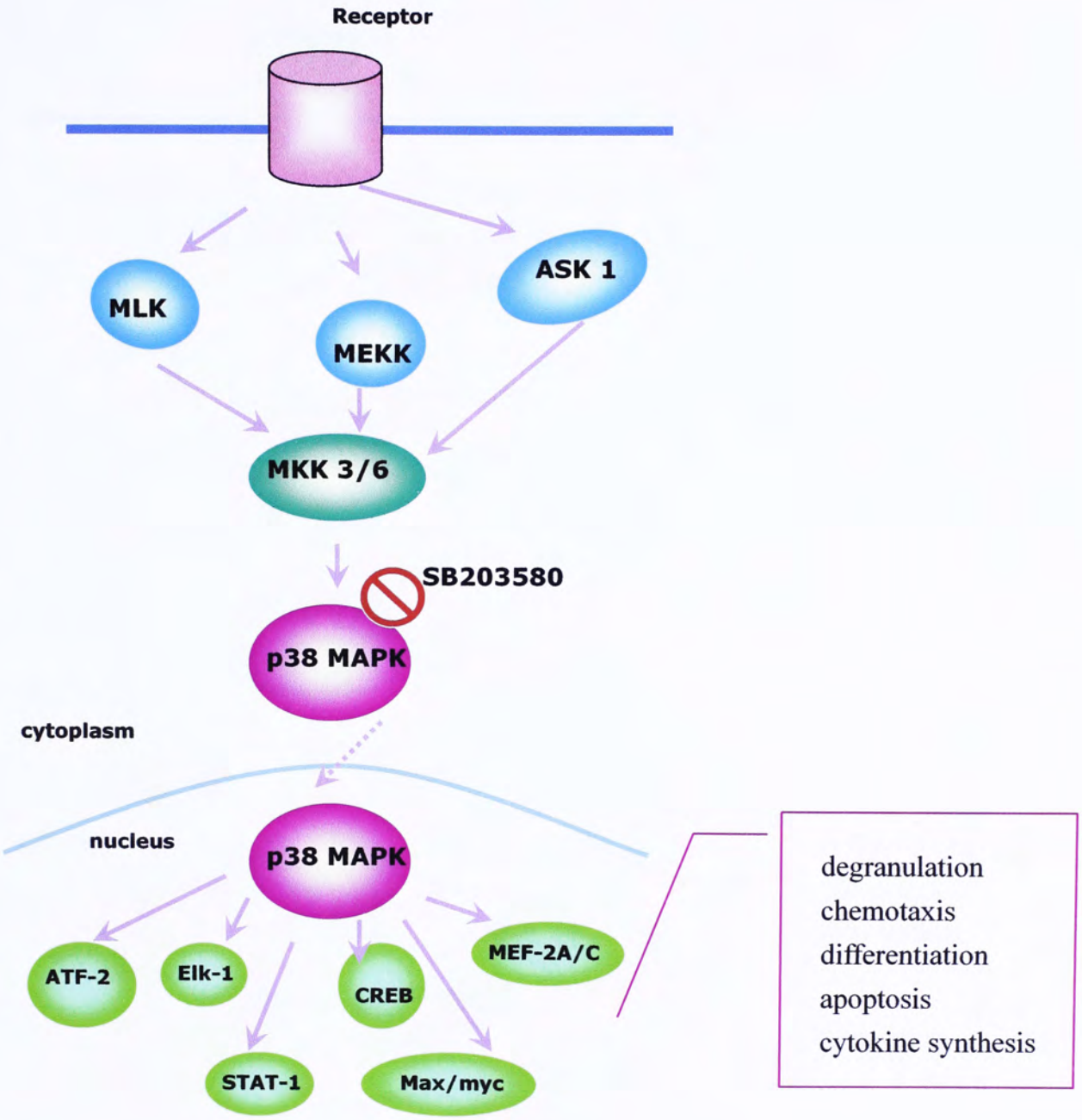


Figure 1.3 The p38 MAPK signaling pathway. SB 203580 is the potent selective p38 MAPK inhibitor.

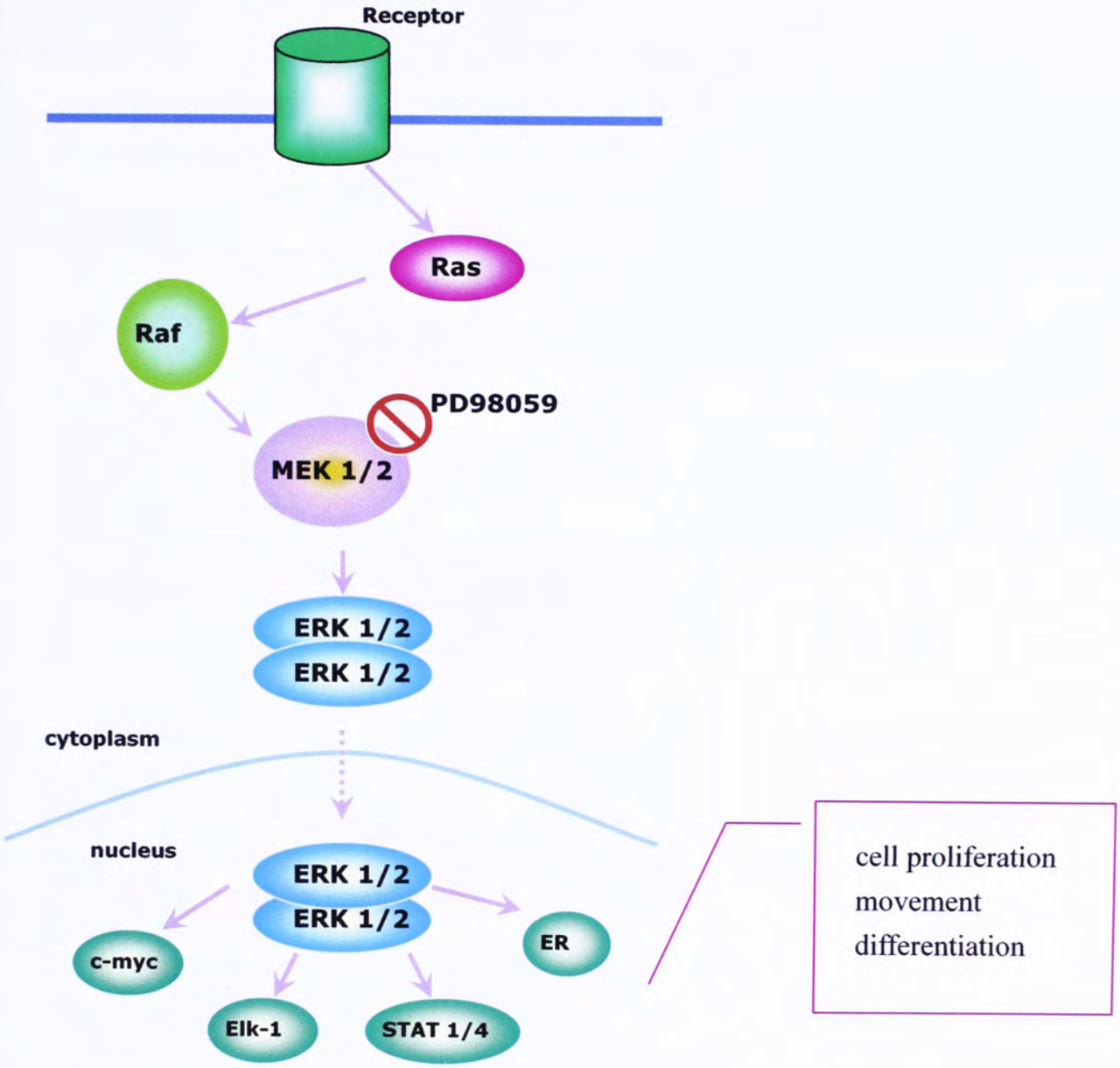


Figure 1.4 The ERK signaling pathway. PD98058 is the selective inhibitor of MEK.

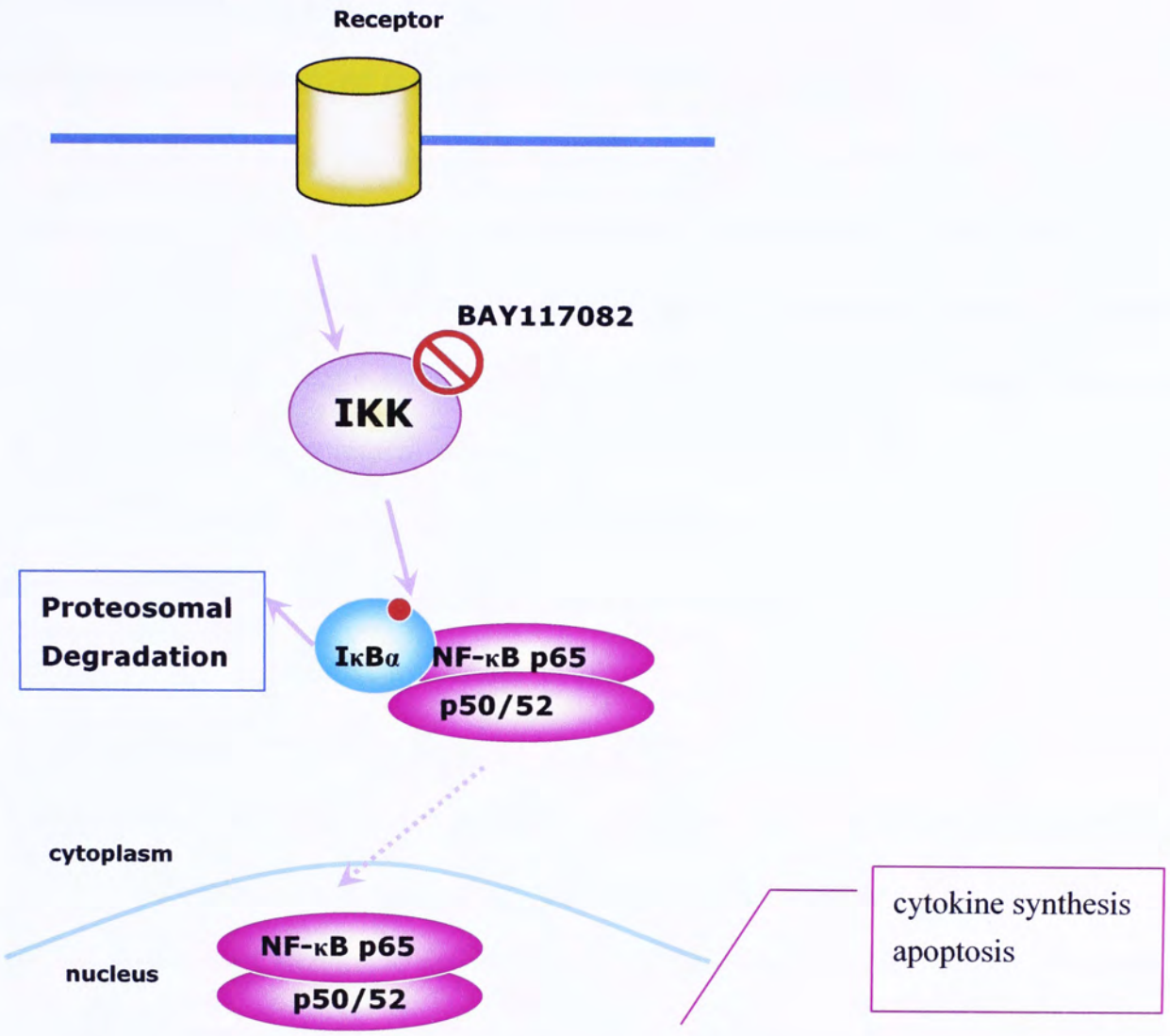


Figure 1.5 The NF-κB signaling pathway. BAY117082 can inhibit the IκB kinase and hence the subsequent phosphorylation of IκB-α

1.5 Signal transduction pathways and pharmacological interventions

As mentioned, p38 MAPK, ERK and NF-κB can act as the key regulators in coordinating genes that control immune responses [Dong et al, 2002; Pomerantz et al, 2002]. ERK and p38 MAPK transduce growth and differentiation signals and mediate inflammatory and stress responses [Kyriakis et al, 2001]. The constitutive activation of NF-κB pathway has been shown to correlate with inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease and allergic asthma [Li et al, 2002]. Intracellular signaling molecules can therefore conduct pathological consequences and inhibition of these pathways may have implications for treating inflammatory diseases [Kowalski et al, 2001].

The past few years have witnessed a remarkable increase in understanding of the details of signaling events stimulated by various cytokines and growth factors. This advance makes pharmacological intervention be possible in different levels throughout signal transduction cascade [Tas et al, 2005]. There are agents which directly affect cytokine or growth factor receptor function by binding and acting as agonists or antagonists or by inhibiting the receptor associated enzymatic activity. Some agents inhibit intermediate steps between the receptor and transcription, including protein kinases and phosphodiesterases [Saklatvala et al, 2004]. Others bind to transcription factors directly and modulate their activity [Young, 1998].

Several pharmacological agents have now been established for showing effects on several signaling pathways. Table 1.2 shows the compounds that can affect signal transduction with the mode of actions and possible therapeutic use (adapted from Young, 1998 and Wong et al, 2004).

Table 1.2. Possible therapeutic use of signaling pathway inhibitors

Molecular target	**compound**	**Mode of action**	**therapeutic use**
p38 MAPK	SB 203580	Inhibit kinase	Anti-inflammatory
MEK	PD 98059 U0126	Inhibit kinase	Cancer metastasis, ischemia, anti-inflammatory
PI3 kinase	Wortmannin	Inhibit kinase	Inhibit neutrophil function
JAK2	Tryphostin	Inhibit kinase	Anti-cancer
TOR	Rapamycin	Inhibit kinase	Immunosuppression
Epo receptor	EMP1	Antagonist	Anemia, acute renal failure
CCR1 receptor	4-hydroxy piperidines	Antagonist	Multiple schlerosis, rheumatoid arthritis
IL-1 receptor	AF12198	Antagonist	Anti-inflammatory
CXCR2 receptor	SB 225002	Antagonist	Reperfusion injury, anti-inflammatory

In future, with the advance in elucidation of the molecular and cellular mechanisms of inflammatory diseases, the signal transduction mechanisms of inflammatory cascades will certainly provide more new potential therapeutic strategies.

1.6 Traditional Chinese medicine (TCM) and pharmacological intervention

In the above section 1.5, we have introduced one way of exploring therapeutic intervention, i.e. inventing chemicals to block certain pathological routes through understanding the molecular mechanisms. However, there is another means to explore efficient treatments through a *vice versa* way; by starting from a substance that has shown efficacy in treating a disease without knowing its mechanisms. TCM has a long history in treating various diseases and been showed to improve general well being by enhancing and modulating immunity. TCM efficacy has been proved by millions of clinical trials started from thousands of years ago when it was firstly administered in human beings. TCM has now become a new trend in pharmaceutical development especially in treating chronic and age-related diseases. Well-planned *in vitro*, animal and clinical research studies have been carrying out by different research groups to elucidate the mechanisms by which TCM exerts it effects on different diseases.

1.6.1 Anti-allergic activities of traditional Chinese medicine

One of the chronic diseases, asthma, has attracted researchers particular attention in revealing the TCM efficacy on its treatment. Asthmatic patients now realize that the two major therapies for asthma, β2-adrenoceptor (β2AR) agonists and inhaled corticosteroids (Barnes, 2002) can lead to metabolic, endocrine and systemic adverse effects in long term use. A survey by the National Asthma Campaign found that 60% of people with moderate asthma and 70% with severe asthma in USA have used complementary and alternative medicine to treat their condition so as to compensate the side effects [Huntley et al, 2000]. TCM is the third most popular choice complementary medicine of both adults (11%) and children (6%) suffering from asthma [Ernst, 1998]. Therefore, it is an urgent need to

demonstrate the anti-allergic effects of TCM, which is thought to be associated with fewer side effects and has been widely prescribed by asthmatic patients, through adequately designed clinical trials or *in vitro* studies.

Recently, many publications have provided evidence of TCM in prevention or treatment of asthma. For example, the antiasthmatic effects of Xiao-qing-long-tang (XQLT, 小青龍湯) appear to be partly mediated by stimulation of β2-adrenoceptors, leading to bronchorelaxation, and that XQLT inhibits the infiltration of eosinophils into the airway [Kao et al, 2001]. Oral administration of Bu-Zhong-Yi-Qi-Tang (補中益氣湯) can suppress IgE antibody production and histamine release in type I allergic reaction in mice immunized intraperitoneally with a mixture of ovalbumin and aluminium hydroxide [Suzuki et al, 1999]. Therapeutic activity of *Cordyceps sinensis* in treating asthma is shown to be related to the modulation of Th1 and Th2 cell functions in bronchial airway by suppressing IL-1β, IL-6 and TNF-α production in bronchoalveolar lavage fluids (BALF) cells [Kuo et al, 2001].

1.6.2 Investigation of the *in vitro* anti-allergic activities of a newly developed Wheeze-Relief Formula

In promoting the modernization of TCM and providing clinical research for drug development, a consortium, led by The Chinese University of Hong Kong with close collaboration from the City University of Hong Kong, The Hong Kong Polytechnic University and The Hong Kong University of Science and Technology, was formed. The consortium adopts an efficacy driven approach and establish an evidence-based scientific model for TCM research. One of the priority projects conducted by this consortium is to prove the efficacy of a selected herbal formula on childhood asthma. In Hong Kong, asthma is the commonest chronic disease affecting children. The prevalence was 6.0% in children between 3 and 10 years of age in Hong Kong and 11% in 13 and 14 years old age Group

[Leung et al, 1997]. In Hong Kong, between 1992 and 1995, the lifetime prevalence of asthma and wheeze in school children increased from 6.6% to 11.2% and from 3.7% to 12.4%, respectively [Leung et al, 1997]. To combat this chronic disease with increasing prevalence, a Wheeze-Relief formula has been designed by Chinese Medicine practitioner. This Wheeze-Relief formula consists of five herbal components, including *Cordyceps sinensis* (冬蟲夏草, Dong Chong Xia Cao), *Bulbus Fritillariae cirrhosae* (川貝, Chuan Bei), *Radix Stemonae* (百部, Bai Bu), *Radix astragali* (黃耆, Huang qi) and *Radix scutellariae* (黃芩, Huang qin). They are common ingredients in many patent Chinese cough mixtures for treating chronic bronchitis. They are also well known to relieve symptoms of many respiratory diseases.

Cordyceps sinensis is traditionally considered as a tonic to nourish the lungs and kidneys. This herb has an antiasthmatic effect by causing smooth muscle relaxation and potentiating the epinephrine effects. It can also exhibit an immunopotentiating effect in treating cancer and immunodeficient patients [Kuo et al, 2001].

Bulbus Fritillariae cirrhosae is a good antitussive agent that is used to relieve coughing and dyspnea, in case of chronic bronchitis or upper respiratory infection. It can cause bronchodilation and inhibition of mucosal secretions [Huang et al, 1999].

Radix Stemonae can suppress excitation of the respiratory center and inhibit the coughing reflex. It also exerts antituberculous, antibacterial and antifungal effects [Liao et al, 1997].

Radix astragali is an important herb in TCM to treat common cold and influenza. The polysaccharide of the herb has antirhinoviral activity and can promote and potentiating interferon functions on antiviral activity [Shon et al, 2002].

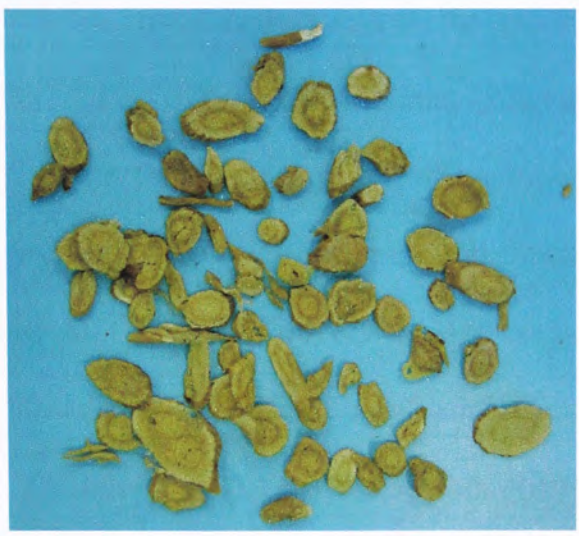
Radix scutellariae has antibacterial and antiviral activity. This herb and its active principle, baicalin, are used in the treatment of upper respiratory infections, such as acute tonsillitis and acute laryngopharyngitis [Liao et al, 2003; and Huang et al, 1999].

In our laboratory, *in vitro* investigation and drug authentication using this Wheeze-relief formula were carried out in the past few years. We have shown that the anti-asthmatic effects may be brought out by immunomodulation on eosinophils and PBMC.

(A)

(B)

(C)



(D)

(E)

Figure 1.6 Raw herbs of (A) *Bulbus Fritillariae cirrhosae* (川貝), (B) *Cordyceps sinensis* (冬蟲夏草), (C) *Radix astragali* (黃耆), (D) *Radix scutellariae* (黃芩) and (E) *Radix Stemonae* (百部).

http://www.hkbu.edu.hk/~cmedyr4/cmedall/cmed.html

1.7 Scope and aims of the study

As mentioned in 1.1.4, mast cells assume multiple roles in body immunity. They are central effector cells of immediate-type hypersensitivity such as allergic asthma and atopic rhinitis. They are also involved in T helper (Th) cells -mediated inflammation such as inflammatory bowel disease [He et al, 2004] and rheumatoid arthritis [Woolley et al, 2003], as well as both natural and acquired immunity [Marone et al, 2002]. An accumulation of mast cells in the inflammatory sites is observed in all the mast cell-mediated inflammatory reactions [Bischoff et al, 2002]. Besides, under the effects of cytokines in the inflamed tissues, mast cells mediate the recruitment of different subpopulation of leukocyte by producing different chemokines [Selvan et al,1994].

Therefore, the regulation of recruitment of mast cells and also their interaction with other inflammatory cells have essential importance in the mechanism of inflammation [Fitzgerald et al, 2004]. However, the underlying mechanisms in recruiting mast cells from the circulation through their cell surface-expressed adhesion molecules are still poorly defined. The interaction of mast cells with other inflammatory cells either through direct intercellular adhesion or paracrine activation is also largely not clarified. To more clearly elucidate the functions of mast cells in inflammation, expression of adhesion molecules and chemokines of human (HMC-1) mast cells after cytokine stimulations was studied.

SCF, TNF-α, IL-13, IL-18 and IL-25, which play key roles in activating mast cells or conducting inflammatory responses (mentioned in 1.2), were used to stimulate HMC-1 cells. The expression of cell surface-expressed adhesion molecules, ICAM-1 and ICAM-3, was then assayed. They have been shown to be associated with mast cell trafficking and transduction of messages to other cells after binding with their ligands (mentioned in 1.3.1). The release of chemokines including IL-8, IP-10, RANTES, MCP-1, I-309 and MIP-1β from HMC-1 cells after the cytokine stimulation was also studied. All these chemokines have been

shown to be released by mast cells and take different roles during inflammation (mentioned in 1.3.2). With the results obtained, we could then more clearly define the biological and pathological roles of mast cells in inflammation. Certain therapeutic strategies in treating mast cell-related diseases may also be devised.

To further understand the mechanisms in regulating the responses of mast cells under the effects of different cytokines, the activation of three intracellular signal transduction pathways including p38 MAPK pathway, ERK pathway and NF-κB pathway was studied. These three signaling pathways have been shown to play important roles in regulating inflammatory responses (mentioned in 1.4). ERK pathway inhibitor PD98059, p38 MAPK pathway inhibitor SB203580 and NF-κB pathway inhibitor BAY117082 were also used to elucidate which signaling pathway is responsible for a specific cytokine-induced response. Apart from getting important information in understanding the pathological consequences mediated by these intracellular signaling molecules, we could also generate pharmacological implications for treating inflammatory diseases.

At last, to further reveal the roles of mast cells in immunity, we used a membrane array to simultaneously profile 96 genes associated with inflammatory responses. The array could give information on the expression of cytokines, chemokines and their receptors on HMC-1 cells. It could then provide much information in deducing the functions of mast cells in inflammations.

Another part of my study was to investigate the *in vitro* anti-allergic activities of a Wheeze-relief formula comprised by five herbal components, including *Cordyceps sinensis*, *Bulbus Fritillariae cirrhosae*, *Radix Stemonae*, *Radix astragali* and *Radix scutellariae.* The results obtained in the first part of study, i.e. the cytokine-induced responses of mast cells, were used to develop immunological parameters in assessing the potential anti-allergic activities of the formula. Experiments were performed to reveal whether the addition of the five herbs could or could not suppress the effects of cytokines on HMC-1 cells.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 HMC-1 cell Line

HMC-1 cells were a generous gift from Dr. J. H. Butterfield of the Mayo Clinic, Rochester, MN, USA. It is a human mast cell line established from the peripheral blood of a patient with mast cell leukemia.

2.1.2 Media and reagents for cell culture

(1) Culture medium

Iscove's Modified Dulbecco's Medium (IMDM) containing L-glutamine and supplemented with sodium bicarbonate at pH 7.2 was used to culture HMC-1. This culture medium was purchased from Gibco Invitrogen Corp, CA, USA.

(2) Serum supplements

Fetal calf serum (FCS) was purchased from Hyclone Co, MA, USA. It had been tested with low endotoxin (<10 EU/ml) and hemoglobin (<10 mg/ml) level. Heat-inactivated fetal calf serum (HI-FCS) was prepared as 50 ml aliquots by heating at 56°C for 30 minutes and stored at -20°C until use.

(3) Phosphate-buffered-saline (PBS) solution

Sterilized PBS solution (0.2 g potassium chloride, 8 g sodium chloride and 1.15 g dibasic sodium phosphate in 1 L) at pH 7.4 was purchased from Gibco Invitrogen Corp.

(4) Endotoxin-free solution

Cell culture medium which was free of detectable LPS (<0.1 EU/mL) was purchased from Gibco Laboratories, Grand Island, NY, USA. All other solutions were prepared using pyrogen-free water and sterile polypropylene plasticware. No solution contained detectable LPS, as determined by the Limulus amoebocyte lysate assay (sensitivity limit 12 pg/ml; Cambrex Bio Science Walkersville Inc, MD, USA)

2.1.3 Recombinant human cytokines

(1) Recombinant human SCF and TNF-α

SCF and TNF-α (Pepro Tech EC Ltd, London, UK) were a lyophilized recombinant protein derived from *Escherichia coli* (*E. coli*) with specific activity of ≥ 2 x 10^7 units/mg. It was reconstituted in autoclaved distilled water to 20 μg/ml in sterilized IMDM medium supplemented with 2% HI-FCS. It was kept as 50 μl aliquots at –80°C until use.

(2) Recombinant human IL-13, IL-18 and IL-25

IL-13, IL-18 and IL-25 were purchased from Medical & Biological Laboratories Co Ltd, Nagoya, Japan. It was a lyophilized recombinant protein derived from *E. coli*. Each cytokine was reconstituted 10 μg/ml in sterilized PBS supplemented with 0.5%

BSA. Twenty μl aliquots were kept at –80°C until use.

2.1.4 Signal transduction pathway inhibitors: PD98035, SB203580 and BAY117082

These were purchased from Calbiochem, San Diego, California, USA. SB 203580•HCl is a pale yellow solid which is a water-soluble form of the potent selective p38 MAP kinase inhibitor. PD98058 is a pale yellow solid which is a selective and cell permeable inhibitor of MAP kinase kinase (MEK) that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrate. BAY117082 is an off-white crystalline solid that can selectively and irreversibly inhibit the IκB kinase and hence the subsequent phosphorylation of IκB-α, resulting in a decreased expression of NF-κB. PD98058 and BAY117082 were dissolved in dimethyl sulfoxide (DMSO). In all studies, the concentration of DMSO was 0.1 % (vol/vol). SB 203580 was reconstituted in distilled water to 10 mM. Aliquots of inhibitors were stored at –80°C until use.

2.1.5 Monoclonal antibodies and reagents for immunofluorescent staining

(1) Monoclonal antibodies (mAb)

FITC-conjugated mouse IgG_1 anti-human ICAM-1 (CD54) mAB (clone BBIG-I1) was purchased from R & D System Inc, MN, USA. FITC-conjugated mouse IgG_{2b} anti-human ICAM-3 (CD50) mAB (clone TU41), rat IgG_{2a} immunoglobulin isotype control and mouse IgG_1 immunoglobulin isotype control were purchased from BD Pharmingen Corp, CA, USA.

(2) Human Serum

Human serum for blocking procedure in immunofluorescent staining was obtained from venous blood of Chinese healthy volunteers.

(3) FACSFlow sheath fluid

It is a balanced electrolyte solution containing sodium chloride, potassium chloride, disodium EDTA, sodium fluoride and anti-microbial agent. The solution was purchased from BD Bioscience Corp, CA, USA.

(4) FACS medium

The buffer contains 0.5% BSA and 0.01% sodium azide (NaN_3) in 1X PBS. It was used for washing in immunofluorescent staining.

(5) 1% paraformaldehye

Paraformaldehyde powder (10 g) (Sigma Chemical Co, MO, USA) was dissolved in 1 L 1X PBS for cell fixation.

2.1.6 Reagents and buffers for chemokine detection

(1) Cytometric Bead Array (CBA) kit

Chemokine Human CBA kit I for measurement of IL-8, RANTES, MIG, MCP-1 and IP-10, was purchased from BD Pharmingen Corp. The kit contains cytometer setup beads, PE positive control detector, FITC positive control detector for compensation settings. It also contains five populations of capture beads, PE conjugated detection reagents, standards and wash buffer for chemokine detection.

(2) ELISA for I-309 and MIP-1β

The ELISA kits were purchased from R & D Systems Inc. These assays employ the quantitative sandwich enzyme immunoassay technique. The kits contain either a microplate pre-coated with monoclonal antibody specific for I-309 or MIP-1β, a secondary antibody that is conjugated to horseradish peroxidase and also a tetramethylbenzidine (TMB) substrate.

2.1.7 Reagents and buffers for Total RNA Extraction

(1) DNase/RNase-Free DEPC-treated Distilled Water

Double distilled water was treated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma Chemical Co), and shaken thoroughly to disperse the DEPC. After standing overnight, the solution was then autoclaved at 121°C for 15 minutes.

(2) Qiagen RNeasy® Total RNA Extraction Kit

The kit purchased from Qiagen GmbH, Hilden, Germany, was used to extract RNA. It consisted of a guanidine isothiocyanate containing lysis buffer RLT for cytolysis, RNeasy spin columns for RNA capture, wash buffers RW1 and RPE for RNA precipitation and washing, and RNase-free water for RNA elution. Each RNeasy spin column had a binding capacity of 100 μg.

(3) β-Mercaptoethanol (2-ME)

Stock solution of 2-ME was purchased from Sigma Chemical Co. In 1ml lysis buffer of Qiagen RNeasy® total RNA extraction kit, 10 μl of 2-ME was added prior to cell lysis.

(4) RNase-Free DNase Set

The DNase set (Qiagen GmbH) contained RNase-Free DNase I (2500 Kunitz units/mg), RNase-Free buffer and water. It was used for on-column digestion of contaminated genomic DNA during RNA purification using RNeasy Total RNA extraction kit.

2.1.8 Reagents and buffers for Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

(1) First-strand cDNA synthesis kit

The kit was purchased from Amersham Biosciences Corp and stored at -20 °C. It consisted of the following components:

a. Bulk First-Strand cDNA Reaction Mixes: Cloned, FPLCpure® Murine Reverse Transcriptase, RNAguard (porcine), RNase/DNase-Free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer.

b. DTT Solution: 200 mM aqueous solution.

c. pd(N)6 Primer: Random hexadeoxynucleotides at 0.2 μg/μl.

d. Not I-d$(T)_{18}$ Bifunctional Primer: 5' d[AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}]-3' at 5 μg/μl.

e. mRNA Standard: Synthetic mRNAs; 1 μg in aqueous solution at 50 μg/ml.

f. DEPC-treated RNase-Free Water

(2) AmpliTaq Gold™ with GeneAmp® 10X PCR buffer and $MgCl_2$ solution

AmpliTaq Gold DNA polymerase (250 units, 5 U/μl), 10 × PCR reaction buffer [100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin] and

25 mM $MgCl_2$ solution were all from Perkin Elmer Corp, CA., USA, and stored at -20 °C.

(3) Primers

The PCR primers specific for different human genes were purchased from Gibco Invitrogen Corp. The lyophilized primers were reconstituted in TE buffer to obtain a stock at a concentration of 100 pmoles/μl. The stocks were aliquoted and kept at –20°C. The primer sequences, annealing temperatures, and their PCR product sizes were as follows:

Human ICAM-1

Sense: 5'-CGT GCC GCA CTG AAC TGG AC-3'

Anti-sense: 5'-CCT CAC ACT TCA CTG TCA CCT-3'

Annealing temperature: 60°C

PCR product size: 447-bp

Human IL-8

Sense: 5'-CTG TGT GAA GGT GCA GTT TTG CC-3'

Anti-sense: 5'-CTC AGC CCT CTT CAA AAA CTT CTC C-3'

Annealing temperature: 55°C

PCR product size: 237-bp

Human IP-10

Sense: 5'- CCT GCT TCA AAT ATT TCC CT -3'

Anti-sense: 5'- CCT TCC TGT ATG TGT TTG GA -3'

Annealing temperature: 55°C

PCR product size: 229-bp

Human MCP-1

Sense: 5'- AAT GCC CCA GTC ACC TGC TGT TAT -3'

Anti-sense: 5'- GCA ATT TCC CCA AGT CTC TGT ATC -3'

Annealing temperature: 55°C

PCR product size: 427-bp

Human RANTES

Sense: 5'- ATA TTC CTC GGA CAC CAC AC -3'

Anti-sense: 5'- CAC GTC CAG CCT GGG GAA GG -3'

Annealing temperature: 55°C

PCR product size: 370-bp

Human I-309

Sense: 5'-GCC CAA GCC AGA CCA GAA GAC A -3'

Anti-sense: 5'-AAG CAG GGC AGA AG AAT GGT G -3'

Annealing temperature: 65°C

PCR product size: 403-bp

Human MIP-1β

Sense: 5'-TAC CAT GAA GCT CTG CGT GAC T -3'

Anti-sense: 5'- ATT AAG AGA AGG GAC AGG AAC T-3'

Annealing temperature: 55°C

PCR product size: 400-bp

Human β-actin

Sense: 5'-AGC GGG AAA TCG TGC GTG-3'

Anti-sense: 5'-CAG GGT ACA TGG TGG TGC C-3'

Annealing temperature: 60°C

PCR product size: 300-bp

(4) PCR nucleotide mix

It was a premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP, each at a concentration of 10 mM in water at pH 7.5. The mixture was purchased from Promega Corp, Madison, WI, USA and stored at -20 °C.

(5) TE Buffer

This consisted of 10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA in distilled water. The solution was used for cDNA dilution and kept at room temperature.

2.1.9 Reagents and Buffers for Protein Extractions

(1) RIPA cell lysis buffer

It was bought from Assay Designs Inc, MI, USA and contains 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 20 mM sodium pyrophosphate, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS and

1 X protease inhibitors. The cell lysis buffer was used to extract total proteins for Western blot analysis and kept as 5 ml aliquots at –80°C.

(2) NE-PER™ Nuclear and cytoplasmic extraction kits

This was purchased from Pierce Biotechnology Inc, IL, USA. It supplied a complete set of cytoplasmic and nuclear extraction reagents that enabled the separation of non-denatured and active proteins of nuclear extract and cytoplasmic fraction from cultured cells and tissue. It contains cytoplasmic extraction reagent I (CER I) and cytoplasmic extraction reagent II (CER II) for lysing the cell membranes and release of cytoplasmic contents and nuclear extraction reagent (NER) for lysing the nuclei. It was stored at 4°C and used to extract nuclear protein for NF-κB activity assay.

2.1.10 Reagents and buffers for detection of activated signaling pathways

(1) Micro BCA Protein Assay Kit

The kit was purchased from Pierce Biotechnology Inc and used to determine the concentration of proteins.

(2) Enzyme-Linked-Immunosorbent-Assay (ELISA) kit for phospho-ERK and phospho-p38

The ELISA kit were purchased from Assay Designs Inc. and for quantifying the phospho-ERK and phospho-p38 in the cell supernatant. Either monoclonal antibody to ERK or p38 immobilized are coated on a microtiter plate. The primary antibody is a rabbit polyclonal antibody to phospho-ERK or phospho-p38. The conjugate antibody is goat anti-rabbit IgG conjugated to horseradish peroxidase.

(3) BD Mercury™ TransFactor Kits

The kit was purchased from BD Biosciences Corp. It is a ELISA-based kit that can detect the quantity of NF-κB. Oligonucleotides containing the consensus binding sequences for a variety of transcription factors are coated on the plate. The bound transcription factors are detected by a specific primary antibody to NF-κB p50. A horseradish peroxidase-conjugated secondary antibody is also included in the kit to detect the bound primary antibody.

(4) p38 MAP kinase assay kit

The kit was purchased from Cell Signaling Technology Inc, MA, USA. It contains all reagents required to measure p38 MAP kinase activity including immobilized phosphorylated-p38 (Thr180/Thr182) monoclonal antibody on agarose G, phosphorylated-ATF-2 (Thr 71) antibody (rabbit polyclonal IgG), ATF-2 fusion protein, kinase buffer, and 10mM ATP.

2.1.11 Reagents and buffers for agarose gel electrophoresis

(1) Ethidium bromide (EtBr) solution

The solution (10 mg/ml) was purchased from Gibco Invitrogen Corp and used for agarose gel electrophoresis of nucleic acids at 0.5 μg/ml.

(2) Tris-acetate-EDTA (TAE) electrophoresis buffer (10X)

TAE buffer (10X Ultra Pure) from Gibco Invitrogen Corp contained 400 mM Tris-acetate and 10 mM EDTA at pH 8.3. It was diluted to 1X TAE buffer in agarose DNA electrophoresis.

(3) 100 base pair DNA ladder marker

100 base pair DNA ladder marker (Gibco Invitrogen Corp) was buffered in TE Buffer at concentration of 1 μg/μl. This reagent was 10-fold diluted with loading buffer and TE buffer, and 5 μl of the diluted DNA ladders was used per lane.

(4) Agarose gel

Agarose gels (1% and 2%) were prepared by dissolving 1g and 2g agarose (Gibco Invitrogen Corp) respectively in 100 ml TAE buffer (0.5X) by heating in microwave. The 1% agarose gel was used for RNA gel electrophoresis while the 2% agarose gel was used for DNA and PCR products gel electrophoresis.

2.1.12 Reagents and buffers for SDS-polyacrylamide gel electrophoresis (PAGE)

(1) 30% (w/v) acrylamide solution

The 30% acrylamide/Bis solution (29:1) was purchased from Bio-Rad Laboratories, CA, USA. It was light-protected and stored at 4°C.

(2) 10% (w/v) ammonium persulfate (APS) solution

This was prepared by dissolving 0.5 g APS (Sigma Chemical Co) in 5 ml double distilled water. The solution was kept in 500 μl aliquots at –20°C until use.

(3) Tris-HCl buffer (pH 7.5)

Tris[hydroxylmethyl] amino methane (Tris) from Sigma Chemical Co was prepared as 1 M stock solution in DEPC-treated double distilled water. The buffer was adjusted to pH 7.5 with hydrochloric acid (HCl), autoclaved and stored at 4°C.

(4) Upper buffer for stacking gel and lower buffer for separating gel

The upper buffer was a 0.5 M Tris-HCl buffer adjusted to pH 6.8 and the lower buffer was a 1.5 M Tris-HCl buffer adjusted to pH 8.8. Both buffers were kept at 4°C.

(5) 10% sodium dodecyl sulfate (SDS) solution

This was prepared by dissolving 10 g SDS (Sigma Chemical Co) in 100 ml double distilled water and kept at room temperature.

(6) N,N,N',N'-Tetra-methylethylenediamine (TEMED)

TEMED purchased from Sigma Chemical Co, was used to initiate the polymerization of SDS-polyacrylamide gel. It was stored at 4°C in dark.

(7) Tris-Glycine-SDS electrophoresis buffer (10X)

Tris-glycine-SDS electrophoresis buffer 10X concentrate containing 0.25 M Tris-HCl, pH 7.5, 1.92 M glycine and 1% SDS in double distilled water was filtered by a 0.2 μm millipore filter and stored at 4°C. This concentrate was diluted fresh to 1X working buffer solution for SDS-PAGE.

(8) Laemmli sample buffer

Laemmli sample buffer from Bio-Rad, contained 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS and 0.01% bromophenol blue. Before use, 50 μl of 2-ME was added to 950 μl of sample buffer for a final concentration of 5% 2-ME, 710 mM. One part of sample was diluted with two parts of sample buffer.

(9) Prestained SDS-PAGE standards

The prestained SDS-PAGE standards (Bio-Rad Laboratories) contained a blue dye attached to standard proteins including phosphorylase B (104 kDa), bovine serum albumin (81 kDa), ovalbumin (47.7 kDa), carbonic anhydrase (34.6 kDa), soybean trypsin inhibitor (28.3 kDa) and lysozyme (19.2 kDa). The standard proteins (total 625 μg) were prepared in 33% (v/v) glycerol, 3% SDS, 10 mM Tris (pH 7.0), 10 mM DTT, 2 mM EDTA, 0.01% NaN_3, and stored at –20°C.

2.1.13 Reagents and buffers for Western blot analysis

(1) Antibodies

Rabbit anti-phospho-ATF-2 (Thr71) and anti-phospho-IκB-α (Ser32) polyclonal antibodies were supplied by Cell Signaling Technology Inc. Horseradish peroxidase conjugated donkey anti-rabbit Ig secondary antibody was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden.

(2) PVDF Western blotting membranes

The microporous polyvinylidene difluoride (PVDF) membrane (pore size: 0.45 μm) was purchased from Amersham Pharmacia Biotech Ltd, Uppsala, Sweden and was stored at room temperature.

(3) Tris-glycine buffer (10X)

Tris-glycine buffer 10X concentrate consisting of 0.25 M Tris-HCl, pH 7.5 and 1.92 M glycine in double distilled water was filtered by a 0.2 μm millipore filter and stored at 4°C.

(4) Tris-glycine-methanol transfer buffer (1X)

The transfer buffer was prepared by mixing 20% methanol in 1X Tris-glycine buffer, and was kept at 4°C

(5) Tris-buffered saline tween 20 (TBST) washing buffer

This consisted of 10 mM Tris-HCl (pH 7.4), 150 mM sodium chloride and 0.05 % (w/v) Tween 20 (Sigma Chemical Co). The solution was kept at 4°C.

(6) 5 % Non-fat milk solution (blocking solution)

Five grams of non-fat milk powder (San Hua Co, Hong Kong) was freshly dissolved in 100 ml TBST washing buffer. The solution was used as a blocking solution.

(7) ECL Western blotting analysis system

It was a Western blotting detection kit purchased from Amersham Pharmacia Biotech Ltd. The kit contained a detection reagent 1 (62.5 ml), detection reagent 2 (62.5 ml), anti-mouse Ig secondary antibody (horseradish peroxidase-linked whole antibody from sheep, 100 µl), anti-rabbit Ig secondary antibody (horseradish peroxidase-linked whole antibody from donkey, 100 µl), and 5 g blocking reagent. The kit was used for the detection of either mouse or rabbit membrane bound primary antibodies and stored at 4°C.

(8) ECL films (Hyperfilm ™ ECL ™)

The Hyperfilm ™ ECL ™ was a high performance chemiluminescence film used for Western blot analysis. It was purchased from Amersham Pharmacia Biotech Ltd.

2.1.14 Reagents and buffers for cDNA expression array analysis

(1) Non-radioactive GEArray^{TM} kits

GEArray Q-series Human Inflammatory Cytokine/Receptor was purchased from SuperArray Bioscience Corp, Bethesda, MD, USA. Each Q-series array membrane comprised 96 marker genes in quadraplicate, 4 positive controls including β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclophilin A and ribosomal protein L13a, and a negative control, bacterial plasmid pUC18.

(2) RNasin® ribonuclease inhibitor

The enzyme from Promega Corp, WI, USA was supplied in 20 mM HEPES-potassium hydroxide (pH 7.6), 50 mM potassium chloride, 8 mM dithiothreitol (DTT) and 50 % (w/v) glycerol. Its concentration was 40 units/μl, and served to inactivate RNase by non-covalent binding.

(3) Deoxynucleoside triphosphates (dNTPs)

Also from Promega Corp, the package consisted of separated vials containing sodium salt of dATP, dCTP, dGTP, and dTTP at concentration of 100 mM (pH 7.5). It was used to freshly prepare the dNTP mix in cDNA labeling.

(4) Salmon sperm DNA solution

The solution was purchased from Gibco Invitrogen Corp and kept at -20°C. It was prepared from phenol-chloroform extracted salmon sperm DNA with average size of ≤ 2000 bp at concentration of 10 mg/ml in DEPC-treated water. The DNA was used at

concentration of 100 μg/ml in both the prehybridization and hybridization solutions.

(5) Biotin-16--dUTP

This is the tetralithium salt of biotinylated-dUTP at a concentration of 1 mM and from Roche Diagnostics Corp, IN, USA. It was used for cDNA labeling and kept at -20°C.

(6) MMLV-reverse transcriptase

The enzyme from Promega Corp was supplied at a concentration of 200 unit/μl and buffered in 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol. It was kept at -20°C.

(7) 20X SSC solution

20X SSC solution consisted of 3M NaCl and 0.3M sodium citrate at pH 7.0, and was purchased from Gibco Invitrogen Corp. It was diluted to 2X and 0.1X SSC in washing solution for the superarray experiments.

2.1.15 Reagents and buffers for cell viability and proliferation assay

(1) Thiazolyl blue tetrazolium bromide (MTT)

It is purchased from Simga-Aldrich Inc., Missouri, USA. MTT was dissolved in PBS to 5mg/ml and stored at -4°C.

(2) Cell proliferation ELISA, 5-bromo-2'-deoxyuridine (Brdu) (colorimetric)

The kit was purchased from Roche Diagnostics GmbH, Penzerg, Germany. It is a colorimetric immunoassay for the quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis. It consists of BrdU in PBS (10mM, pH7.4), fixing and denaturing solution, anti-BrdU monoclonal antibody conjugated with peroxidase, antibody dilution solution, washing buffer and substrate solution.

2.1.16 Reagent kit for endotoxin level assay

QCL-1000 ® Chromogenic Limulus Amebocyte Lysate (LAL) Test was purchased from Cambrex Bio Science Walkersville Inc, MD, USA. The kit contains the Limulus Amebocyte Lysate (LAL) prepared from the circulating amebocytes of the horseshoe crab *Limulus polyphemus, E. coli* endotoxin and chromogenic substrate.

2.2 Methods

2.2.1 HMC-1 cell cultures

HMC-1 cells were maintained in IMDM at a density between 3 and 7 x 10^5 cells/ml supplemented with 10 % (vol/vol) HI FCS (Gibco Invitrogen Corp), and 1.2 mM α–thioglycerol (Sigma Chemical Co). They were kept under a humidified atmosphere with 5 % CO_2 at 37°C and subcultured every 4 days.

2.2.2 Flow cytometry of cell surface expression of ICAM-1 and ICAM-3

HMC-1 cells (5 x 10^5 cells/0.5 ml) after preceding treatment were harvested and resuspended with cold phosphate buffered saline (PBS) supplemented with 0.5 % bovine serum albumin (BSA). After blocking with 2 % human pooled serum for 20 min at 4°C and washed with PBS supplemented with 0.5 % BSA, cells were incubated either with FITC-conjugated mouse anti-human ICAM-1 and ICAM-3 monoclonal (mAb) antibody or fluorescein-conjugated mouse IgG_1 and IgG_{2a} isotype for 30 min at 4°C in dark. After washing, cells were finally resuspended in 1 % paraformaldehyde in 1X PBS as fixative. Cell surface expression of ICAM-1 and ICAM-3 was then analyzed by flow cytometry (FACSCalibur analyser, Becton Dickinson Biosciences Corp, CA, USA) in terms of mean fluorescence intensity (MFI).

2.2.3 Total cellular RNA extraction

Total RNA from HMC-1 cells (3 x 10^6 cells) was extracted using RNeasy Mini Kit (Qiagen GmbH). Briefly, HMC-1 cells were lysed and homogenized in the presence of a highly denaturing guanidinium isothiocyanate-containing buffer that immediately inactivated RNases to ensure isolation of intact RNA. Ethanol was added to provide appropriate binding conditions and the sample was then applied to an RNeasy mini spin column where the total RNA bound to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in 40 µl of RNase-free water after 15 min on-column DNA digestion.

2.2.4 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Extracted total RNA was reverse transcribed into first-strand complementary DNA using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Ltd) according to instruction of manufacturer. PCR was performed in a reaction mixture containing 3 mM $MgCl_2$, 200 μM dNTPs, 1 unit of AmpliTaq Gold DNA polymerase (Perkin Elmer Corp), 50 pmol of 5' and 3' primers (Gibco Invitrogen Corp) in PCR reaction buffer (30 sec at 94°C for denaturing, 30 sec at annealing temperature specific for each pair of primers and 45 sec at 72°C for elongation) from 25 to 35 cycles for different primers, after an initial 12 min of denaturation at 94°C. All RT-PCR were performed in the linear range of the PCR reaction according to the preliminary experiment. The amount of RNA used was normalized by comparison with the amplification of human β-actin mRNA. After the amplification reaction using PTC-200 DNA Engine™ (MJ Research, Inc., MA, USA), PCR products were electrophoresed on 2 % agarose gel in 1 × TAE buffer (pH 8.0) and stained with ethidium bromide.

2.2.5 Agarose gel electrophoresis

Both 1% and 2% agarose gels (w/v) were prepared in 1X TAE (pH 8.3) containing 0.5 μg/ml ethidium bromide. RNA (0.5 -1.0 μg) or 5 μL of PCR product or 1 μg of 1k base-pair DNA ladder, was mixed with 2 μL of fast dye loading buffer (5X) containing 0.05% (w/v) bromophenol blue, 40% (w/v) sucrose, 0.1 M EDTA (pH 8.0) and 0.5% sodium dodecyl sulfate (SDS). Electrophoresis was performed in a horizontal electrophoretic tank (Hoeffer, Pharmacia Biotech) with an agarose gel immersed in 1 × TAE by applying the mixture into the sample well under constant

voltage of 180V for 20 minutes. The bands were visualized under UV. Images were taken from Gene Genius Gel Documentation System (Syngene Inc., Cambridge, UK).

2.2.6 Quantitative analysis of IL-8, IP-10,MCP-1 and RANTES

HMC-1 cells (6x 10^5 cells/0.3 ml) were treated for 24 h and the concentrations of chemokines in culture supernatant were measured by cytometric bead array (CBA) (BD Pharmingen Corp) using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA). The kits contained five bead populations with distinct fluorescence intensities and they have been coated with capture antibodies specific for different cytokines and chemokines. The bead populations could be resolved in the FL3 channel of flow cytometer. First, 50 µl plasma or culture supernatants were incubated with 50 µl different capture beads mixtures and 50 µl PE-conjugated detection antibodies. The mixture formed a sandwich complex and after 3 h incubation, the capture beads were washed and re-suspended for sample data acquisition using Cell Quest (FACSCalibur). The sample results were finally generated in graphical format using the BD CBA analysis software.

2.2.7 Quantitative analysis of I-309 and MIP-1β

Culture supernatant was collected after treating the HMC-1 cells (6x 10^5 cells/0.3 ml) for 24 h. I-309 and MIP-1β were measured by ELISA for I-309 and MIP-1β (R & D System Inc).

2.2.8 Detection of phosphorylated-ERK and phosphorylated-p38 MAPK

HMC-1 cells (3 x 10^6) after preceding treatment were washed with PBS and lysed in 0.15 ml RIPA cell lysis buffer (Assay Designs Inc). The concentrations of phosphorylated-ERK and phosphorylated-p38 MAPK in cell lysate of HMC-1 cells were quantitated by ELISA using the reagent kits of Assay Designs Inc.

2.2.9 Detection of NF-κB activity

Nuclear proteins of HMC-1 cells (1 x 10^6) were extracted with NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce Chemical Co) according to the manufacturer instructions. Nuclear extracts were subjected to NF-κB protein/NF-κB oligonucleotide binding using Mercury™ TransFactor NF-κB p50 kit (BD Biosciences Corp) for the determination of NF-κB activity.

2.2.10 Detection of phosphorylated-ATF-2

Total cellular protein from HMC-1 cells (3 x 10^6) after preceding treatment were lysed in 0.15 ml RIPA cell lysis buffer (Assay Designs Inc). p38 MAP kinase kit (Cell Signaling Technology Inc) was used to detect phosphorylated-ATF-2. In brief, the monoclonal phospho-specific antibody to p38 MAP kinase (Thr180/Thr182) was used to selectively immunoprecipitate active p38 MAP kinase from cell lysates. The immunoprecipitate was later incubated with ATF-2 fusion protein in the presence of ATP and kinase Buffer; this allows immunoprecipitated active p38 MAP kinase to

phosphorylate ATF-2. Phosphorylation of ATF-2 was then assessed by Western blotting.

2.2.11 Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE)

A Bio-Rad Mini-PROTEAN apparatus was used for discontinuous polyacrylamide gel electrophoresis. The recipe of the stacking gel was 0.187 M Tris.HCl, pH 6.8, 5% acrylamide, 0.1% SDS, 0.1% APS, 0.1% TEMED, while the lower separating gel was 0.375 M Tris.HCl, pH 8.8, 12.5% acrylamide, 0.1% SDS, 0.1% APS, 0.075% TEMED. The separating gel solution mixed as the above recipe was casted into the vertical gel glass plates of sizes [Inner: 7.3 × 10.2 × 0.1 cm; Outer: 8.3 × 10.2 × 0.1 cm (h × w × d)]. A layer of 0.1 % SDS was laid on the top to assist the formation of a straight line interphase. After the separating gel was set, the 5 % polyacrylamide stacking gel was set with a comb to form ten wells of sizes 1.5 × 0.5 × 0.1 cm (h × w × d). Electrophoresis was carried out using Tris-Glycine-SDS Running Buffer at a constant voltage of 100 for about 100 – 120 min. After electrophoresis, the gel was either used for immunoblotting or stained with Coomassie brilliant blue dye (0.2 % R 250 Coomassie brilliant blue, 50 % methanol, 10 % acetic acid) for 1 h and de-stained with a solution of 5 % methanol and 10 % acetic acid.

2.2.12 Western blot analysis

An equal amount of proteins was subjected to 10% SDS-PAGE before blotting onto a PVDF membrane (Amersham and Pharmacia Biotech Corp, NJ, USA). The

membrane was blocked with 5 % skimmed milk in Tris-buffered saline with 0.05 % Tween 20, pH 7.6 for 1 hr at room temperature, and probed with primary rabbit anti-human phosphorylated-IκB-α or phosphorylated-ATF-2 antibody (Cell Signaling Technology Inc) at 4°C overnight. After washing, membranes were incubated with secondary donkey anti-rabbit antibody coupled to horseradish peroxidase (Amersham and Pharmacia Biotech Corp) for 1 hr at room temperature. Antibody-antigen complexes were then detected using ECL chemiluminescent detection system according to the manufacturer's instructions (Amersham and Pharmacia Biotech Corp).

2.2.13 MTT assay

HMC-1 cells (2×10^5 cells/0.2 ml) were inoculated into a 96-well plate. Various inhibitors at serial concentrations were added to the cells. After 48-h incubation, 50 μg MTT (Sigma Chemical Co) was added to each well and incubated for 2 h. Viable cells uptook MTT and reduced it into dark blue water-insoluble formazan by mitochondrial dehydrogenase which reflected the normal function of mitochondria and cell viability. The cells were then lysed with DMSO (200 μl) to yield the colour solution. The absorbance at OD550nm was measured to quantify the viable cells.

2.2.14 Cell proliferation assay

The cell proliferation rate was assayed with the colorimetric BrdU ELISA (Roche Diagnostics Corp). HMC-1 cells (2×10^5 cells/0.2 ml) were inoculated into a 96-well

plate. Various stimulator at serial concentrations were added to the cells for 24 h. BrdU was then added and incubated with the cells for 4 h. BrdU was incorporated in place of thymidine into the DNA of proliferating cells. By adding the anti-brdU conjugated with peroxidase and substrate tetramethy-benzidine, the color developed thereby directly correlated to the number of proliferating cells.

2.2.15 Hot water extraction of TCM

Powdered extract (5 g) of Dong Chong xia Cao (冬蟲夏草, *Cordyceps sinensis*), Huang qi (黃耆, *Radix astragali*), Bai Bu (百部, *Radix Stemonae*), Chuan Bei (川貝, *Bulbus Fritillariae cirrhosae*) and Huang qin (黃芩, *Radix scutellariae*) were received from Institute of Chinese Medicine, the Chinese University of Hong Kong, Hong Kong. Autoclaved deionized water (30 ml) was added to each of them and subjected to a water bath of 80°C for 2 h. Supernatant was collected after centrifugation at 5000g for 30 min and filtered through 0.2 μm polythersulfone filter. The filtered supernatant was lyophilized and afterwards reconstituted to 100 mg/ml with 1X PBS. The reconstituted TCM was stored in -20°C.

2.2.16 Endotoxin level assay

The endotoxin level of the TCM was assayed by the Chromogenic Limulus Amebocyte Lysate (LAL) Test. TCM in serial concentrations was added to 96-well plate and mixed with the LAL for 10 min at 37°C. The substrate solution was then mixed with the LAL-sample and incubated at 37°C for an additional 6 min before

stopping by stop reagent. In this process, Gram-negative bacterial endotoxin catalyzed the activation of a proenzyme in the LAL. The activated enzyme then catalyzed the splitting of pNA from the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA. The yellow coloured-pNA could be measured photometrically after the reaction was stopped.

2.2.17 cDNA expression array analysis

Non-radioactive GEArray Q-series Human Inflammatory Cytokine/Receptor kits (SuperArray Bioscience Corp) were used to analyze the gene expression profiles on stimulated cells. Briefly, 3 μg total RNA was used as template for biotinylated cDNA probe synthesis. RNA was reverse-transcribed by gene-specific primers with biotin-16-dUTP. Biotinylated cDNA probes were denatured and added to the hybridization solution. The GEArray membrane dotted with cDNA fragments of different specific genes were pre-hybridized at 60°C for 2 hours and then hybridized overnight with the biotinylated cDNA probes. The membranes were then washed and blocked with GEA blocking solution, and incubated with alkaline phosphatase conjugated streptavidin. The hybridized biotinylated cDNA probes were detected by chemiluminescent method using the alkaline phosphatase substrate, CDP-*Star*®. The results were analyzed using Bio-Rad Quantity One™ software (Bio-Rad Laboratories). The relative expression levels of different genes were estimated by comparing its signal intensity with that of internal control β-actin.

2.2.18 Statistical analysis

Data in figures were presented either as histograms plus SD or as mean ± SD in

curves. Differences between groups were assessed by the non-parametric Mann-Whitney rank sum test. A probability $p < 0.05$ was considered significantly different. All analyses were performed using the Statistical software GraphPad Prism (GraphPad Prism for Windows, version 3.00, GraphPad Software, Inc. CA, USA).

Chapter 3
Results

3.1 The effects of cytokines on the expression of ICAM-1 and ICAM-3 on HMC-1 cells

3.1.1. SCF, TNF-α and IL-13 up-regulated ICAM-1 but not ICAM-3 expression on HMC-1 cells

To generate a profile of cytokines that may regulate ICAM-1 and ICAM-3 expression, the effects of SCF (50 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), IL-18 (20 ng/ml) and IL-25 (50 ng/ml) were examined after 48 h incubation with the HMC-1 cells. The optimal dose and incubation time of the above cytokines had been determined previously for significant effect (data not shown). Compared to controls, a slight increase in ICAM-1 expression by SCF but a significant up-regulation of ICAM-1 expression by TNF-α and IL-13 (Figure 3.3A, $p < 0.05$) were observed. However, IL-18 and IL-25 showed no significant effect on ICAM-1 expression (Figure 3.1A). As shown in Figure 3.1B, ICAM-3 showed a much higher basal expression level, but all cytokines were not able to upregulate its expression.

3.1.2. SCF, TNF-α and IL-13 up-regulated the mRNA expression of ICAM-1

As shown in Figure 3.2, SCF, TNF-α and IL-13 but not IL-18 and IL-25 could up-regulate the mRNA expression of ICAM-1 after 24 h incubation.

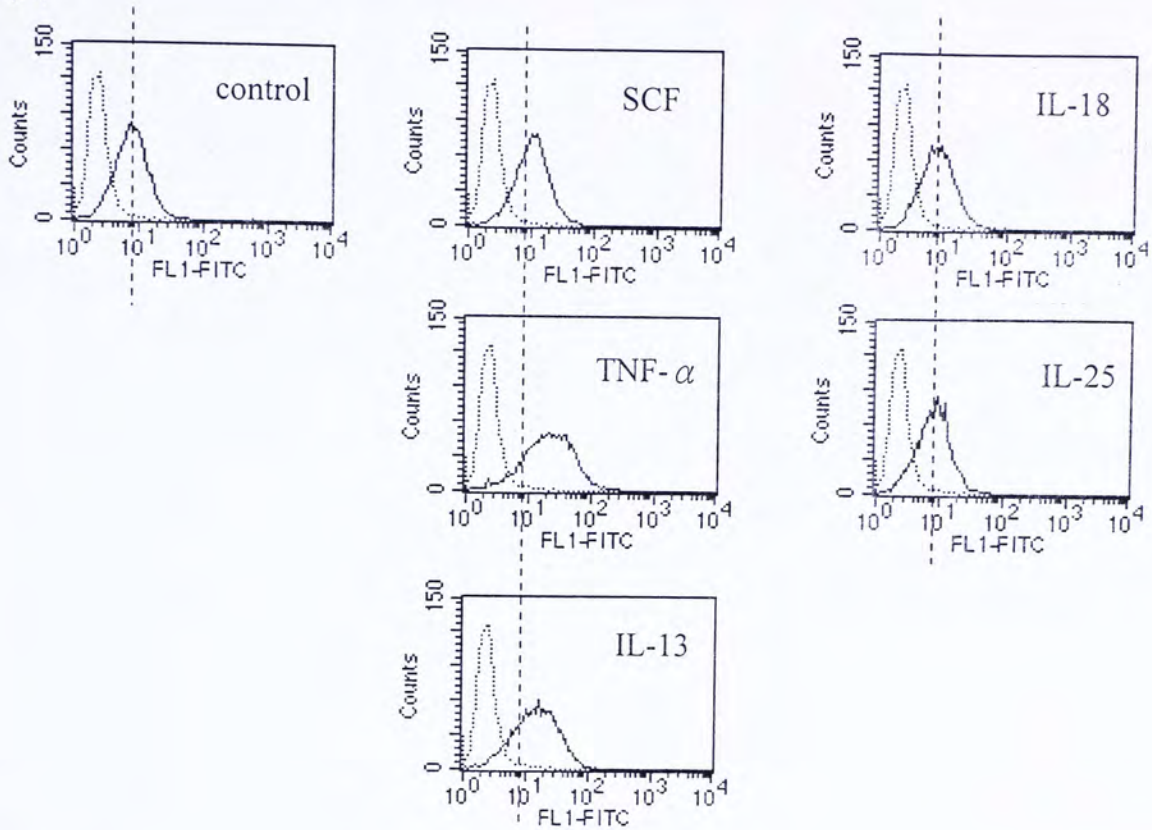
3.1.3 The combined treatment of SCF and TNF-α, and SCF and IL-13 showed synergistic and additive effect on ICAM-1 expression respectively

Figure 3.3 shows that the combined treatment of SCF and TNF-α resulted in a synergistic up-regulation of ICAM-1 expression. However, the combined treatment of SCF and IL-13 was found to have only an additive effect on ICAM-1 expression.

3.1.4 Synergistic up-regulation of ICAM-1 expression in combined treatment of SCF and TNF-α was dose-dependently enhanced by SCF

To investigate whether the synergistic effect was dose-dependent on SCF or TNF-α, serial concentrations of SCF and TNF-α were used in the combined treatment. As shown in Figure 3.4, fixed concentration of SCF (50 ng/ml) with serial concentrations of TNF-α (5 - 70 ng/ml) showed that the synergistic effect could not be dose dependently enhanced by TNF-α. On the other hand, the synergistic effect could be further enhanced when the SCF concentration increased from 10 ng/ml to 100 ng/ml in combination with fixed TNF-α concentration (20 ng/ml).

(A)


control
SCF
IL-18
TNF-α
IL-25
IL-13
Counts
FL1-FITC

(B)

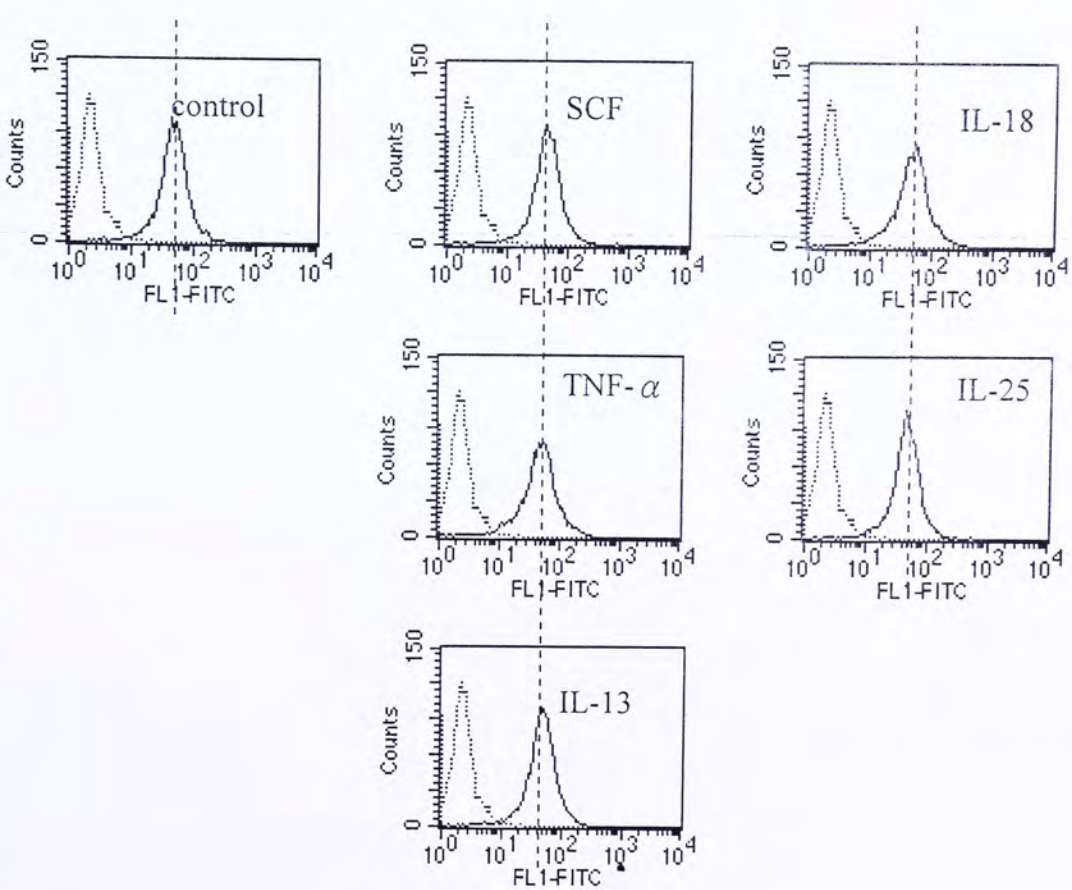

control
SCF
IL-18
TNF-α
IL-25
IL-13
Counts
FL1-FITC

Fig. 3.1. Effects of SCF, TNF-α, IL-13, IL-18 and IL-25 on ICAM-1 and ICAM-3 expression on HMC-1 cells. HMC-1 cells (1 x 10^6 cells/ml) were incubated for 48 h without any cytokines, or with SCF (50 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), IL-18 (20 ng/ml), or IL-25 (50 ng/ml). Staining of HMC-1 cells (A) with mAb IgG_1 isotype control antibody (dotted line) and mAb against ICAM-1 (solid line) and (B) mAb IgG_{2a} (dotted line) and ICAM-3 (solid line) was determined by flow cytometry. Data are presented as histograms of relative cell counts with fluorescence intensity. Each histogram represents 5 x 10^3 cells. These are representative figures from 5 independent experiments with very similar results. Vertical bar indicates cutoff for peak of ICAM-1 or ICAM-3 staining with control medium.

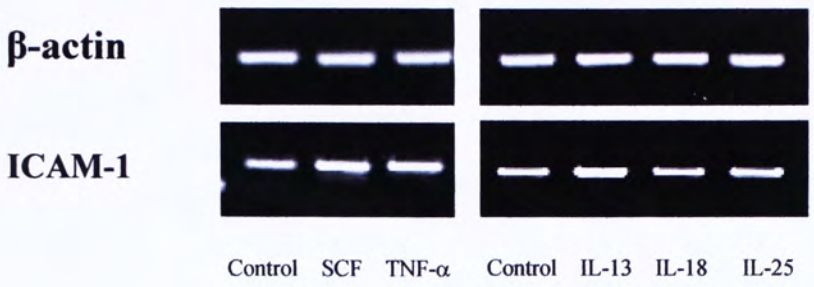


Fig. 3.2. Effects of SCF, TNF-α, IL-13, IL-18 and IL-25 on gene expression of ICAM-1 in HMC-1 cells. mRNA expression of β-actin and ICAM-1 of HMC-1 (1×10^6 cells/ml) after treatment with SCF (50 ng/ml), TNF-α (20 ng/ml), IL-13 (20 ng/ml), IL-18 (20 ng/ml) and IL-25 (50 ng/ml) for 24 h were analysed by RT-PCR. Representative results are shown from triplicates experiments with essentially identical results.

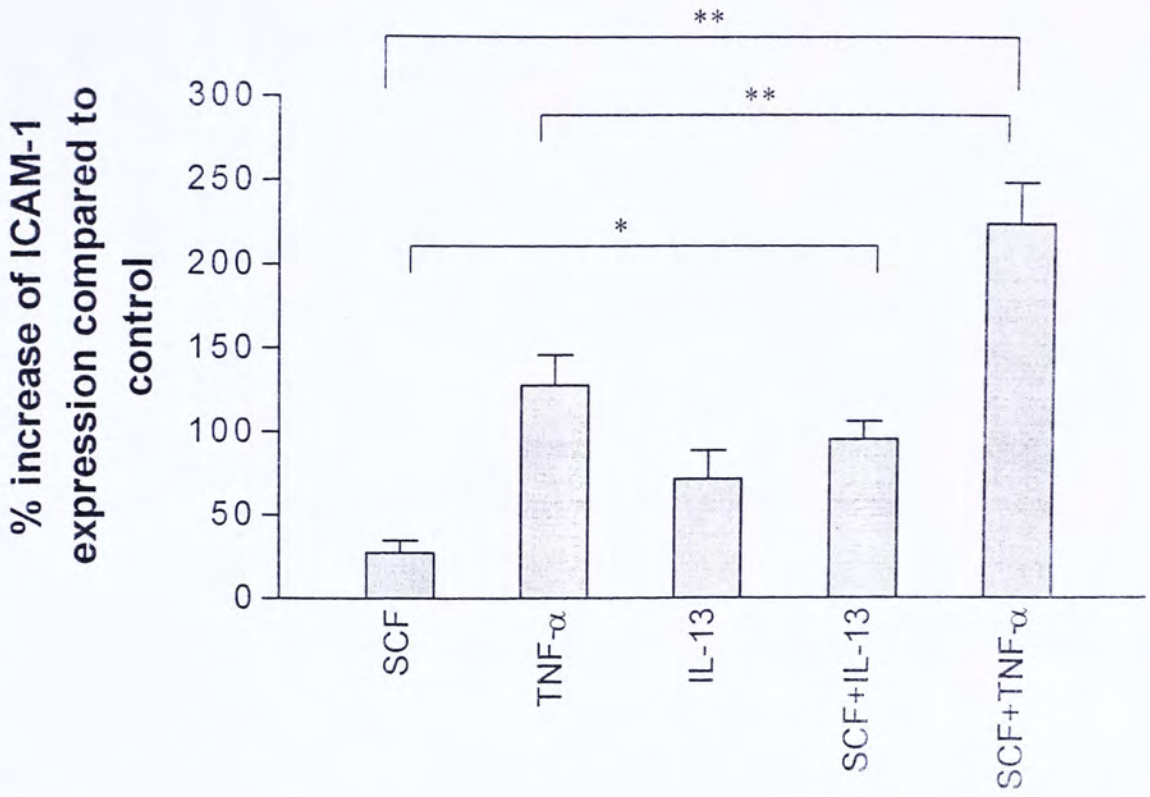


Fig. 3.3. Effects of SCF, TNF-α, IL-13, (SCF + TNF-α) and (SCF + IL-13) on the ICAM-1 expression on HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) were incubated for 48 h without or with SCF (50 ng/ml), TNF-α (20 ng/ml) and IL-13 (20 ng/ml). Percentage increase of ICAM-1 expression was calculated as: [MFI after cytokine treatment - MFI without treatment]/MFI without treatment × 100%. Results are presented as mean plus SD of 7 independent experiments. Mann-Whitney rank sum test was used to assess the difference of % increase between single treatment control groups and combined treatment group. * $p < 0.05$, ** $p < 0.01$.

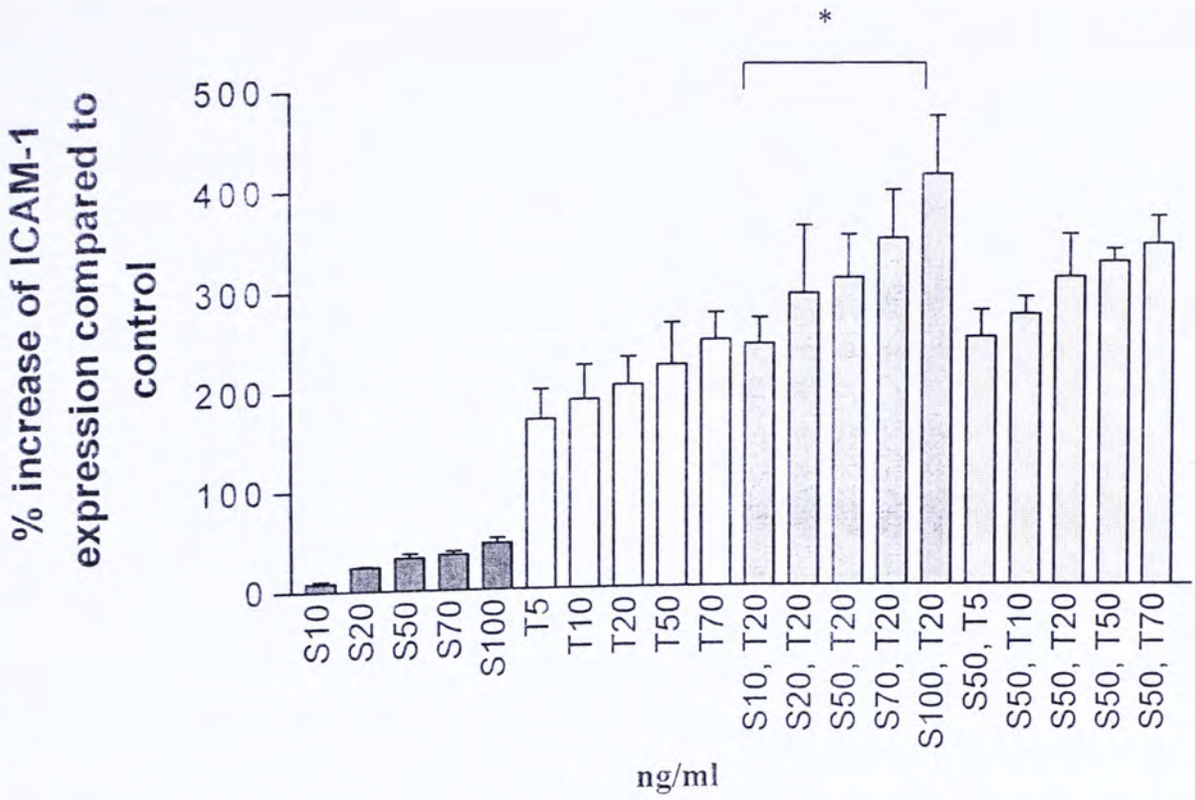


Fig. 3.4. Effects of various concentrations of SCF and TNF-α on the expression of ICAM-1 on HMC-1 cells. HMC-1 cells (1 x 10^6 cells/ml) were treated with SCF (10 - 100 ng/ml) and TNF-α (5 - 70 ng/ml) for 48 h. In the combined treatment, a fixed concentration of either SCF (50 ng/ml) or TNF-α (20 ng/ml) was used with a serial concentration of TNF-α or SCF. $^*p<0.05$ for the combined treatment of SCF (100 ng/ml) + TNF-α (20 ng/ml) compared with the combined treatment of SCF (10 ng/ml) + TNF-α (20 ng/ml) assessed by Mann-Whitney rank sum test. No significant difference was found between the combined treatments of TNF-α (5 - 70 ng/ml) and SCF (50 ng/ml). Results are expressed as mean plus SD of 5 independent experiments.

S: SCF

T: TNF-α

3.2 The effects of cytokines on the release of IL-8, IP-10, MCP-1, RANTES, I-309 and MIP-1β from HMC-1 cells

3.2.1 SCF induced the release of IL-8, MCP-1, RANTES, I-309 and MIP-1β

As shown in Figure 3.5, SCF could significantly induce IL-8, MCP-1, RANTES, I-309 and MIP-1β at 24 h incubation from HMC-1 cells (all $p < 0.05$). SCF could also up-regulate their mRNA gene expression as shown in Figure 3.6. β-actin was used as positive control and remained constant in all treatments.

3.2.2 TNF-α induced the release of IL-8, IP-10, MCP-1, RANTES and I-309

TNF-α could significantly induce IL-8, IP-10, MCP-1, RANTES and I-309 at 24 h incubation from HMC-1 cells (Figure 3.5, all $p < 0.05$). TNF-α could also up-regulate their mRNA gene expression as shown in Figure 3.6.

3.2.3 SCF and TNF-α did not enhance the proliferation rate of HMC-1

To ensure that the increased release of chemokines was not due to an increased cell number under the effect of SCF and TNF-α, a proliferation assay using BrdU was performed. Figure 3.7 shows that the SCF and TNF-α did not significantly increase the proliferation rate of HMC-1.

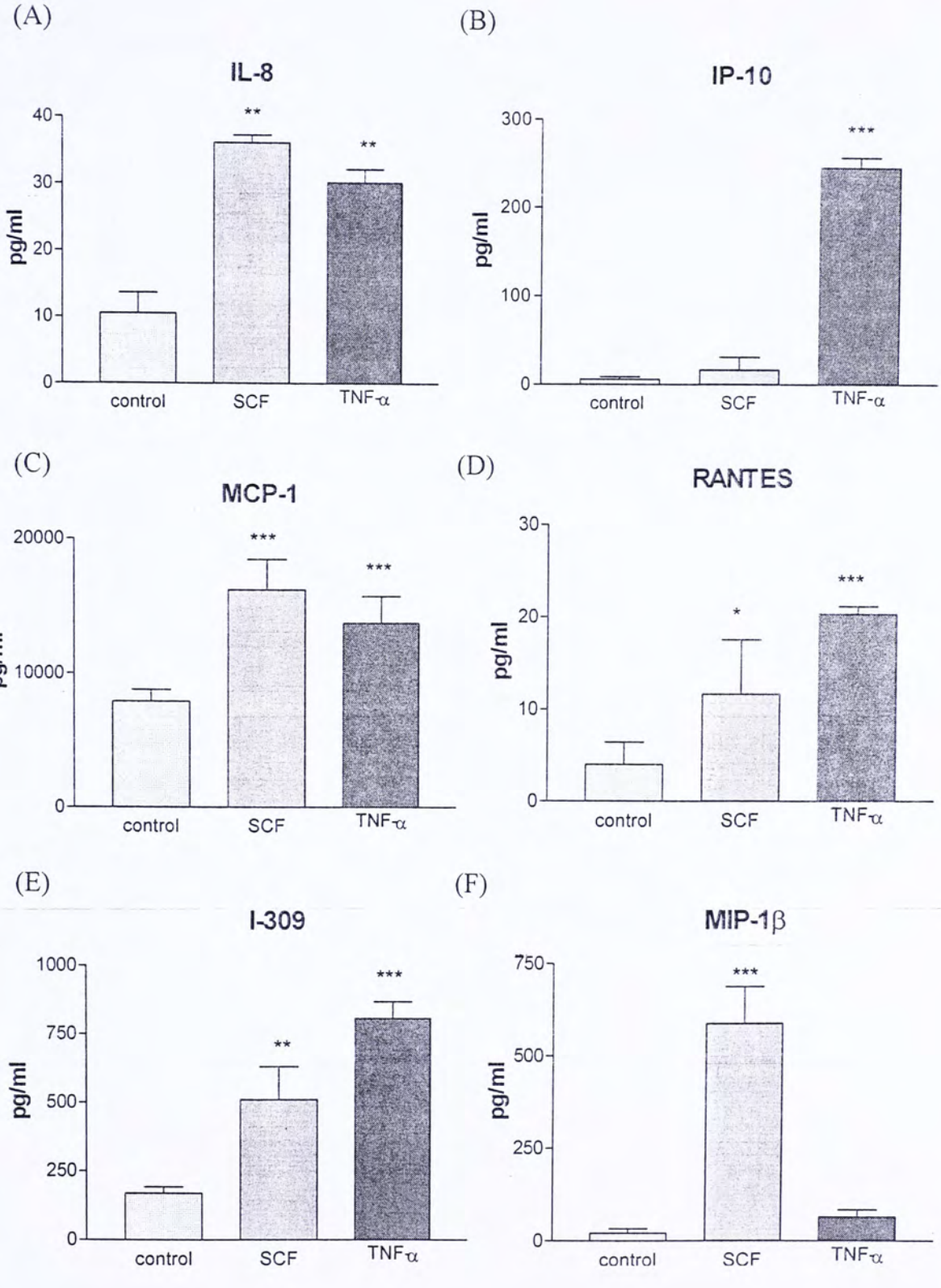

(A)
IL-8
pg/ml
40
30
20
10
0
**
**
control
SCF
TNF-α
(B)
IP-10
pg/ml
300
200
100
0
***
control
SCF
TNF-α
(C)
MCP-1
pg/ml
20000
10000
0
***
***
control
SCF
TNF-α
(D)
RANTES
pg/ml
30
20
10
0
*
***
control
SCF
TNF-α
(E)
I-309
pg/ml
1000
750
500
250
0
**
***
control
SCF
TNF-α
(F)
MIP-1β
pg/ml
750
500
250
0
***
control
SCF
TNF-α

Fig. 3.5. Effect of SCF and TNF-α on the release of (A) IL-8, (B) IP-10, (C) MCP-1, (D) RANTES, (E) I-309 and (F) MIP-1β from HMC-1 cells. HMC-1 cells (2×10^6 cells/ml) were cultured with or without SCF (50 ng/ml) or TNF-α (20 ng/ml) for 24 h in a 24-well plate. IL-8, IP-10, MCP-1, RANTES, and I-309 and MIP-1β released into the culture supernatant were determined by human chemokine CBA kit using flow cytometry and ELISA respectively. Results are expressed as the arithmetic mean plus SD from four independent experiments. Mann-Whitney rank sum test was used to assess the difference between all treatment groups. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ when compared with the control.

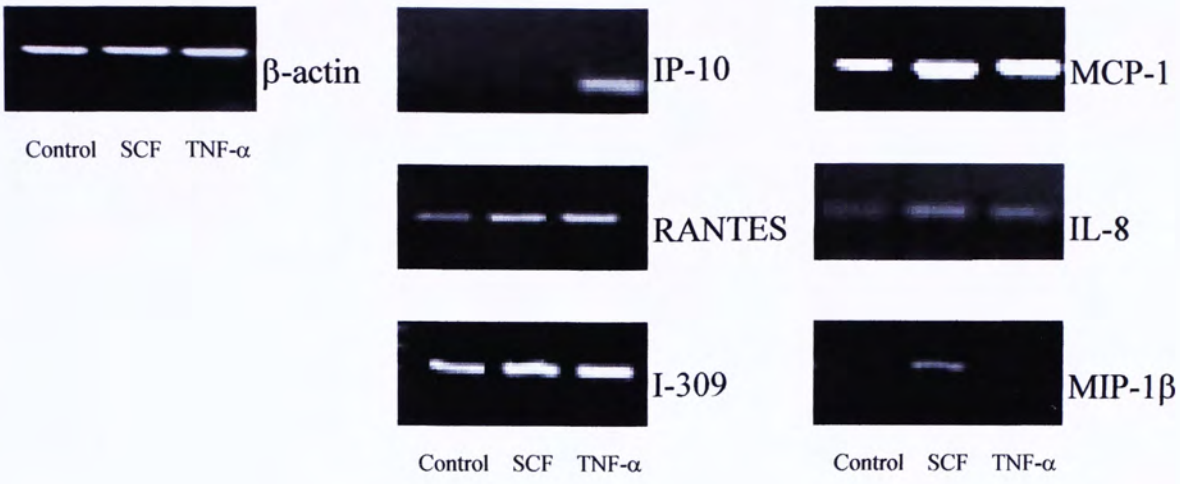


Fig. 3.6. Effects of SCF and TNF-α on gene expression of IL-8, IP-10, MCP-1, RANTES, I-309 and MIP-1β in HMC-1 cells. Total RNA was extracted from HMC-1 cells (1 x 10^6/ml) after treated with or without SCF (50 ng/ml) or TNF-α (20 ng/ml) for 12 h, and then reverse transcribed and analyzed by PCR. The β-actin housekeeping gene was used as the control.

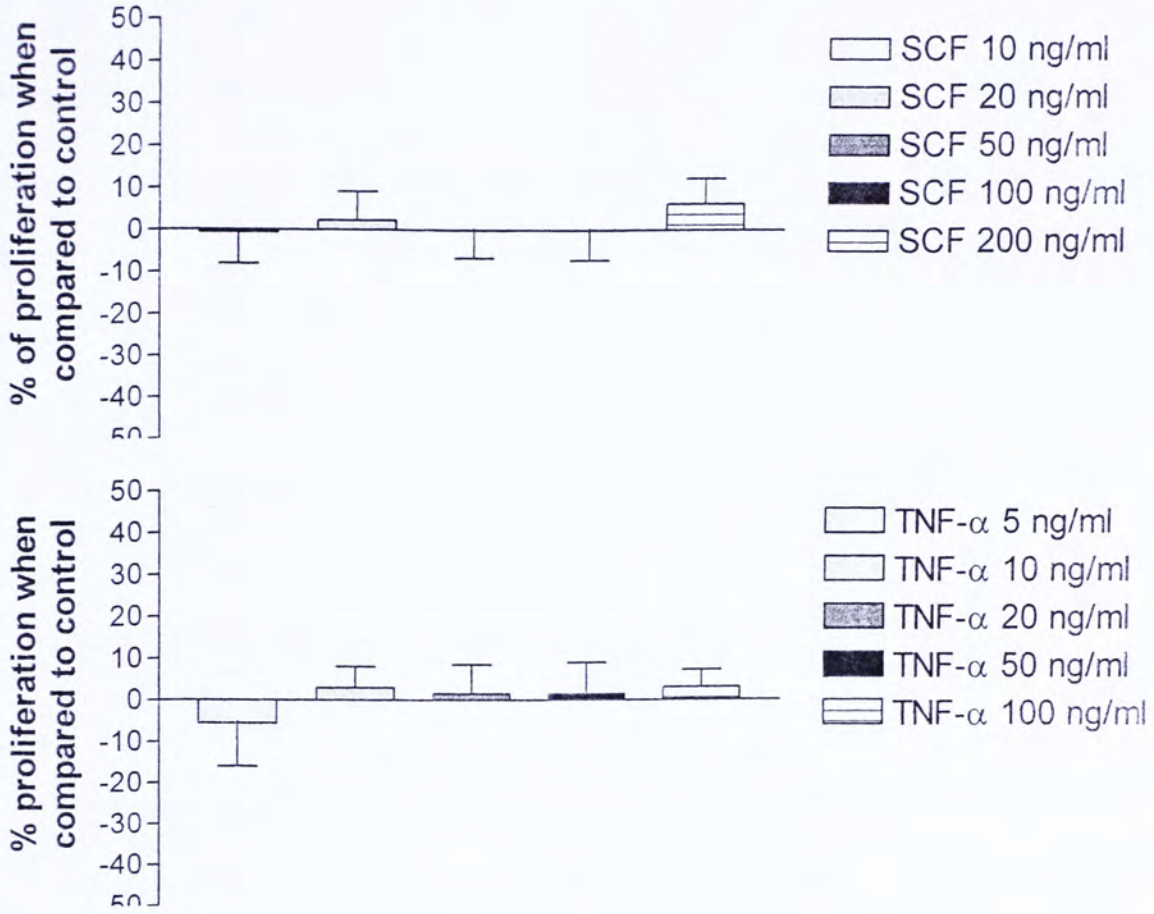


Fig. 3.7. Effects of SCF and TNF-α on the proliferation rate of HMC-1. HMC-1 cells (2 x 10^6 cells/ml) were treated without or with SCF (10 - 200 ng/ml) and TNF-α (5 -100 ng/ml) for 24 h. The proliferation rate was determined by BrdU ELISA. Percentage of proliferation when compared to control was calculated as: absorbance value of treated cells/ absorbance value of control cells X 100%.

3.3 The effects of SCF and TNF-α on the activation of ERK, p38 MAPK and NK-κB

Since SCF and TNF showed significant effect on ICAM-1, IL-8, IP-10, MCP-1, RANTES, I-309 and MIP-1β on HMC-1 cells, the activation of ERK, p38 MAPK and NK-κB signaling pathways were studied to elucidate the intracellular regulatory mechanism. Total cellular protein extracted from HMC-1 cells after cytokine stimulation was used for the determination of phosphorylated p38 MAPK and phosphorylated ERK; while extracted nuclear protein was used for ELISA of NF-κB protein/NF-κB oligonucleotide binding assay.

3.3.1 SCF activated ERK but not p38 MAPK and NF-κB

SCF could activate ERK (Figure 3.8) but not p38 MAPK (Figure 3.9) and NF-κB (Figure 3.10B). The SCF activated ERK pathway peaked at 30 min and declined afterwards (Figure 3.8). It was also observed that TNF-α did not cause any phosphorylation of the ERK (Figure 3.8).

3.3.2 TNF-α activated p38 MAPK and NF-κB but not ERK

In Figure 3.9, it was shown that TNF-α caused activation of p38 MAPK reaching peak level at 15 min and declined afterwards. Results of Western blot (Figure 3.10A) showed that the treatment of TNF-α could induce the degradation of IκB-α, thereby releasing the NF-κB for translocation into nucleus. ELISA of NF-κB protein/NF-κB oligonucleotide binding (Figure 3.10B) showed that the peak level of nuclear translocated NF-κB protein occurred at 7 h after treatment of TNF-α or combined treatment of TNF-α and SCF. Afterwards, level of NF-κB protein binding declined, but was found to be higher in the

combined treatment of SCF and TNF-α than that of treatment of TNF-α at 18 h.

(A)

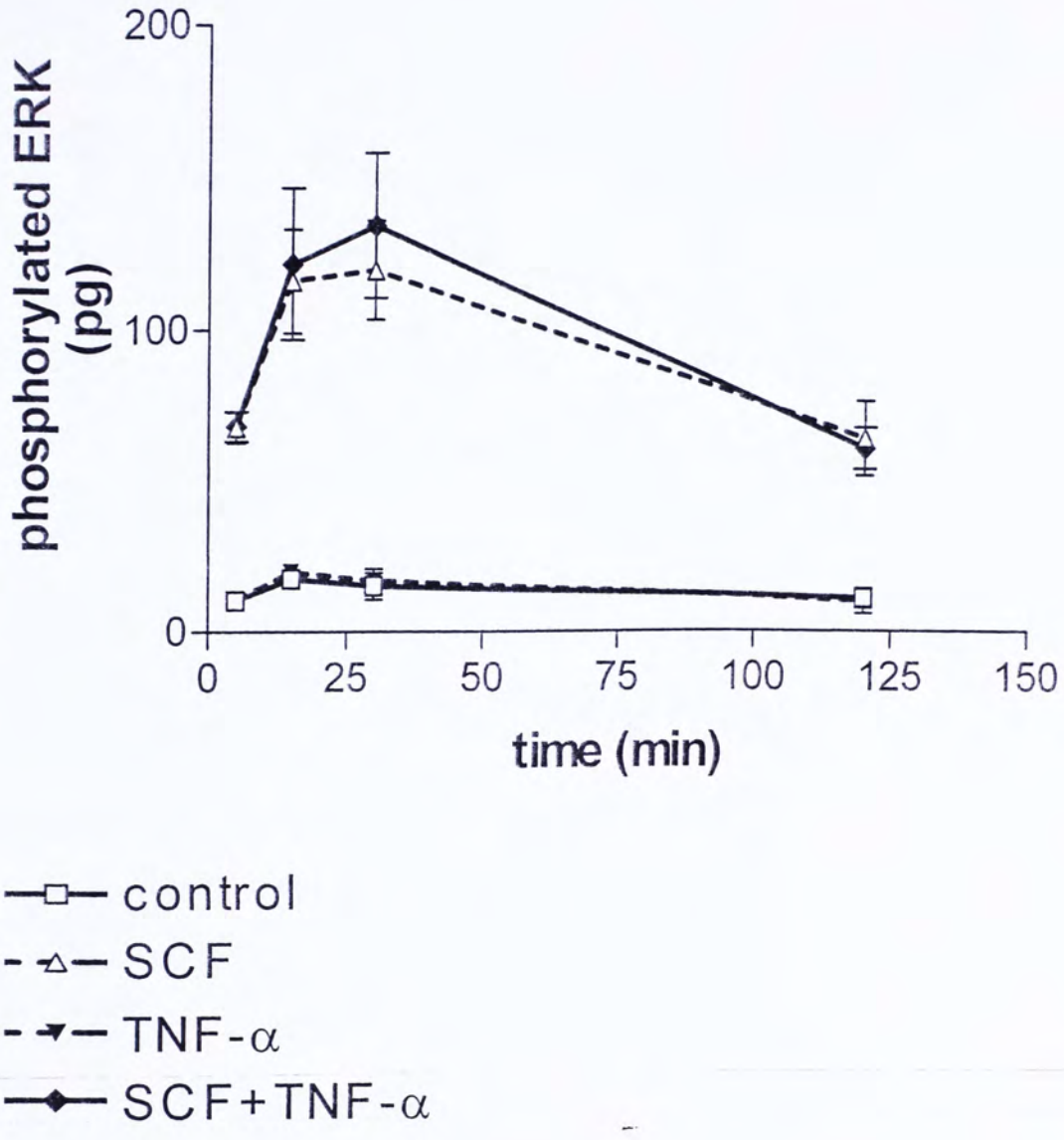


Fig. 3.8. Effects of SCF, TNF-α and SCF+TNF-α on the activation of ERK of HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) were stimulated for 5, 15, 30 and 120 min with SCF (50 ng/ml), TNF-α (20 ng/ml) and combined treatment of SCF+TNF-α. Extracted total cellular protein (10 ng) from treated and non-treated (control) HMC-1 cells were used for the ELISA of phosphorylated-ERK. Results are expressed as mean ± SD of six independent experiments.

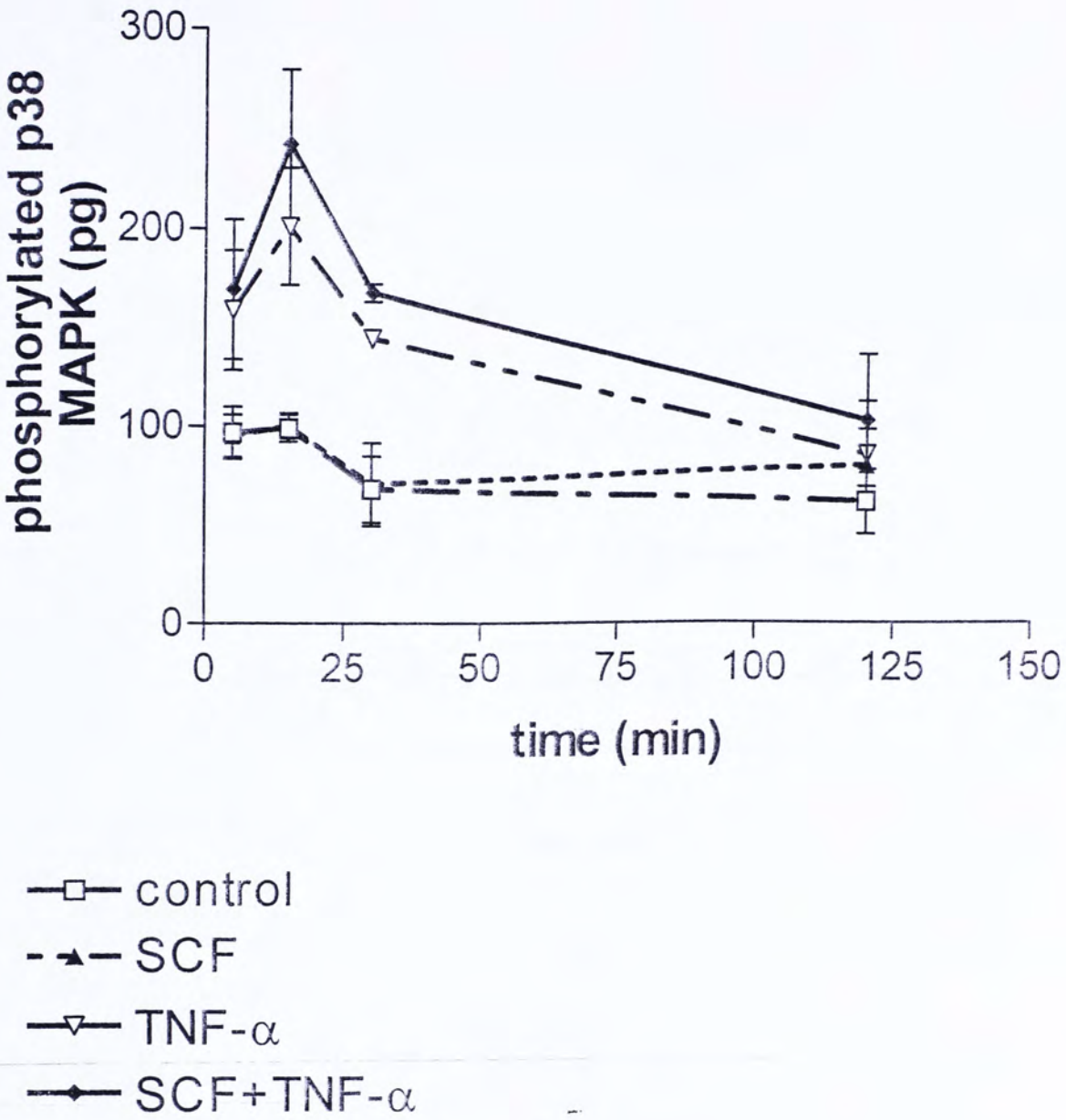


Fig. 3.9. Effects of SCF, TNF-α and SCF+TNF-α on the activation of p38 MAPK of HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) were stimulated for 5, 15, 30 and 120 min with SCF (50 ng/ml), TNF-α (20 ng/ml) and combined treatment of SCF+TNF-α. Extracted total cellular protein (10 ng) from treated and non-treated (control) HMC-1 cells were used for the ELISA of phosphorylated-p38 MAPK. Results are expressed as mean ± SD of six independent experiments.

(A)

Control SCF TNF-α SCF+TNF-α

(B)

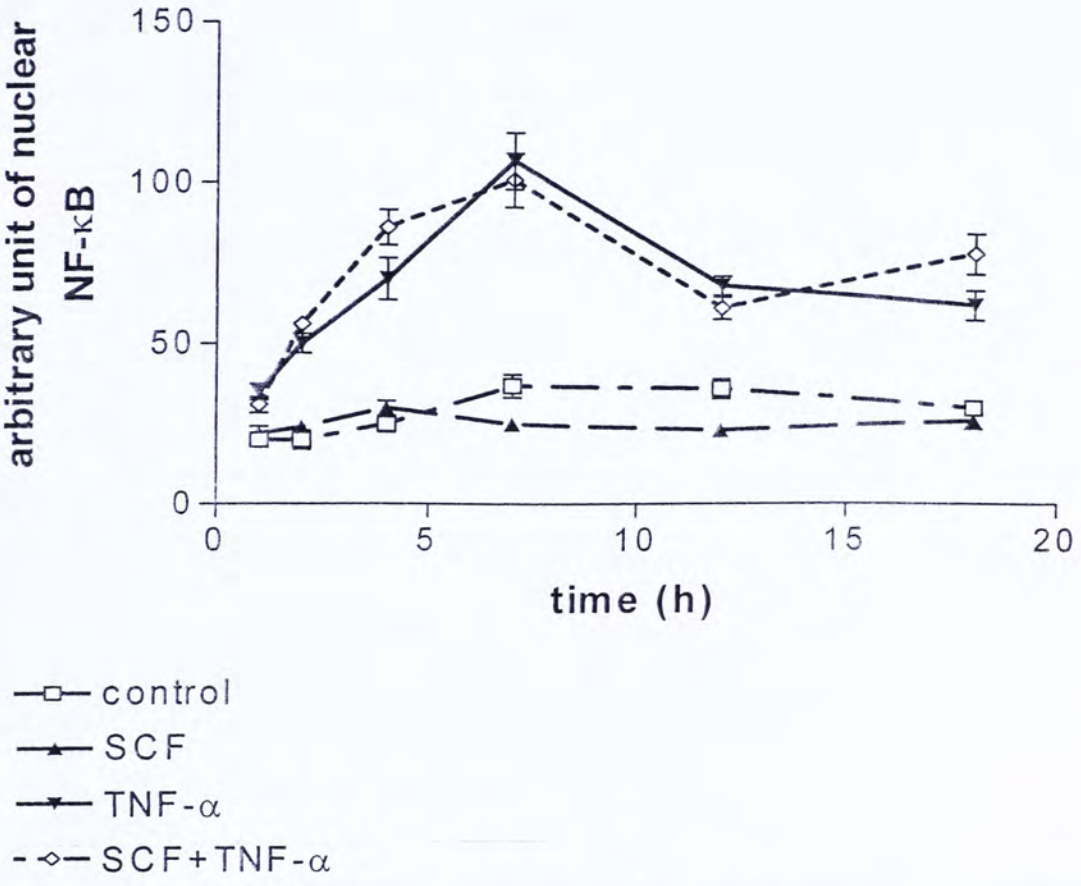


Fig. 3.10. Effects of SCF, TNF-α and SCF+TNF-α on the NF-κB activity of HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) were treated without or with SCF (50 ng/ml), TNF-α (20 ng/ml) and combined treatment of SCF+TNF-α. In Figure (A), extracted total cellular protein (30 μg) after 2 h treatment was used for the detection of phosphorylated IκB-α by Western blot analysis. Representative blot is shown from triplicate experiments with essentially identical results. In Figure (B), extracted nuclear protein (30 μg) after treatment for 1, 2, 4, 7, 12 and 18 h was used for the ELISA of NF-κB binding to NF-κB oligonucleotide. Results are expressed as mean ± SD of six independent experiments.

3.4 The effect of inhibitors of intracellular signal transduction molecules on the SCF and TNF-α-induced release of chemokines

To elucidate which signaling pathway is responsible for the SCF- and TNF-α-induced chemokine release, ERK pathway inhibitor PD98059, p38 MAPK pathway inhibitor SB203580 and NF-κB pathway inhibitor BAY117082 were used for investigating their suppressive effect on the release of chemokines.

3.4.1 The optimal dose of PD98059, SB203580 and BAY117082

The cytotoxicity of different inhibitors for different signaling pathways of HMC-1 cells was firstly determined. The dose at which at least 80 % cells were viable was used as the optimal dosage of the inhibitor. As shown in Figure 3.11, the optimal dose was found to be 50, 20 and 70 μM for PD98059, SB203580 and BAY117082 respectively. DMSO (1 μl/ml) was used as vehicle for PD98059 and BAY117082 and it did not have significant cytotoxic effect on HMC-1 cells (Figure 3.11).

3.4.2 PD98059 suppressed the SCF induced IL-8, MCP-1, RANTES, I-309 and MIP-1β release from HMC-1 cells.

As shown in Figure 3.12, PD98059 (50 μM) could significantly suppress SCF induced release of IL-8, MCP-1, RANTES, I-309 and MIP-1β from HMC-1 cells (all $p < 0.05$).

3.4.3 SB203580 and BAY117082 differentially suppressed the TNF-α induced chemokine release from HMC-1 cells.

In Figure 3.13, SB 203580 (20 μM) could significantly suppress TNF-α-induced IL-8, IP-10, MCP-1 and I-309 while BAY 117082 (70 μM) could suppress IP-10 and RANTES release from HMC-1 cells (all $p < 0.05$).

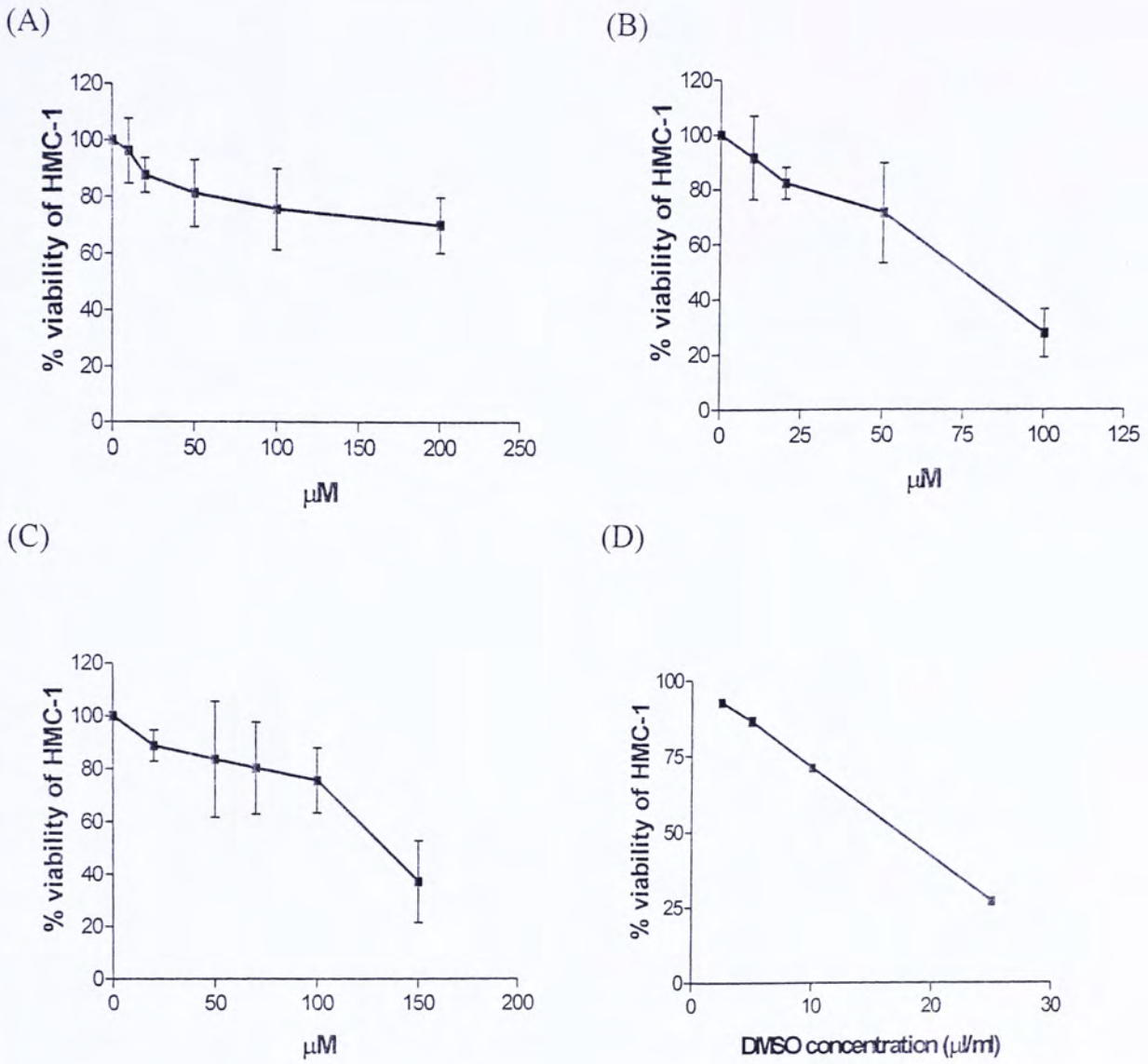


Fig. 3.11. Effects of PD98059, SB203580 and BAY117082 on the viability of HMC-1 cells. HMC-1 cells were incubated with (A) PD98059, (B) SB203580, (C) BAY117082 and (D) DMSO for 48 h and the viability was assessed by MTT assay. DMSO was used as vehicle for inhibitors PD98059 and BAY117082. Results are expressed as mean ± SD of triplicate experiments.

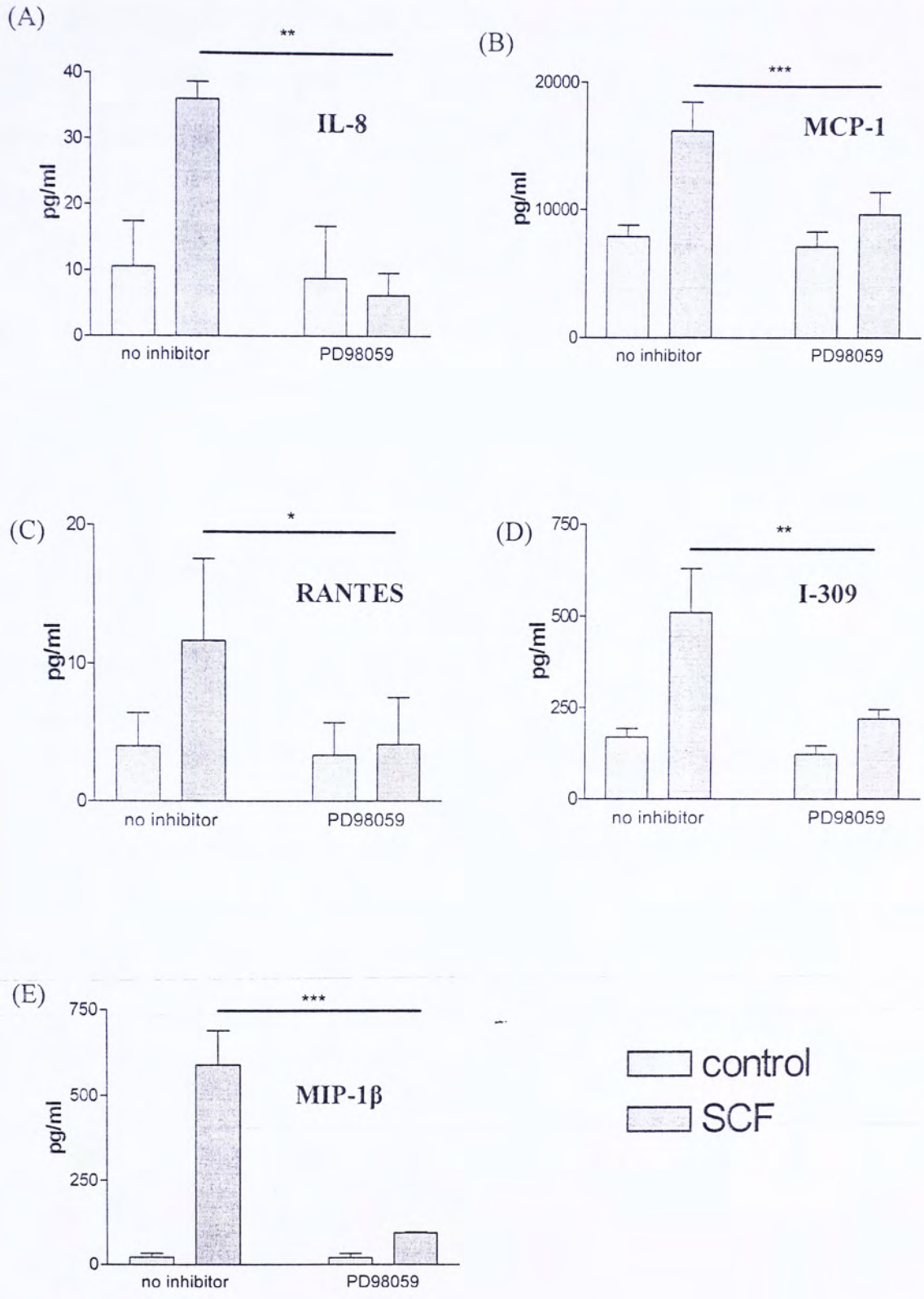

(A)
**
IL-8
pg/ml
40
30
20
10
0
no inhibitor
PD98059
(B)
***
MCP-1
pg/ml
20000
10000
0
no inhibitor
PD98059
(C)
*
RANTES
pg/ml
20
10
0
no inhibitor
PD98059
(D)
**
I-309
pg/ml
750
500
250
0
no inhibitor
PD98059
(E)
***
MIP-1β
pg/ml
750
500
250
0
no inhibitor
PD98059
control
SCF

Fig. 3.12. Effects of PD98059 on the SCF-induced (A) IL-8, (B) MCP-1, (C) RANTES, (D) I309 and (E) MIP-1β in HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated without or with PD98059 (50 μM) for 1 h followed by stimulation with SCF for 24 h. The flow cytometry based CBA was used to detect IL-8, MCP-1 and RANTES, and ELISA was used to detect I-309. Mann-Whitney rank sum test was used to assess the difference between inhibitor-treated group and the non-inhibitor-treated group. Results are expressed as mean plus SD of triplicate experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$.

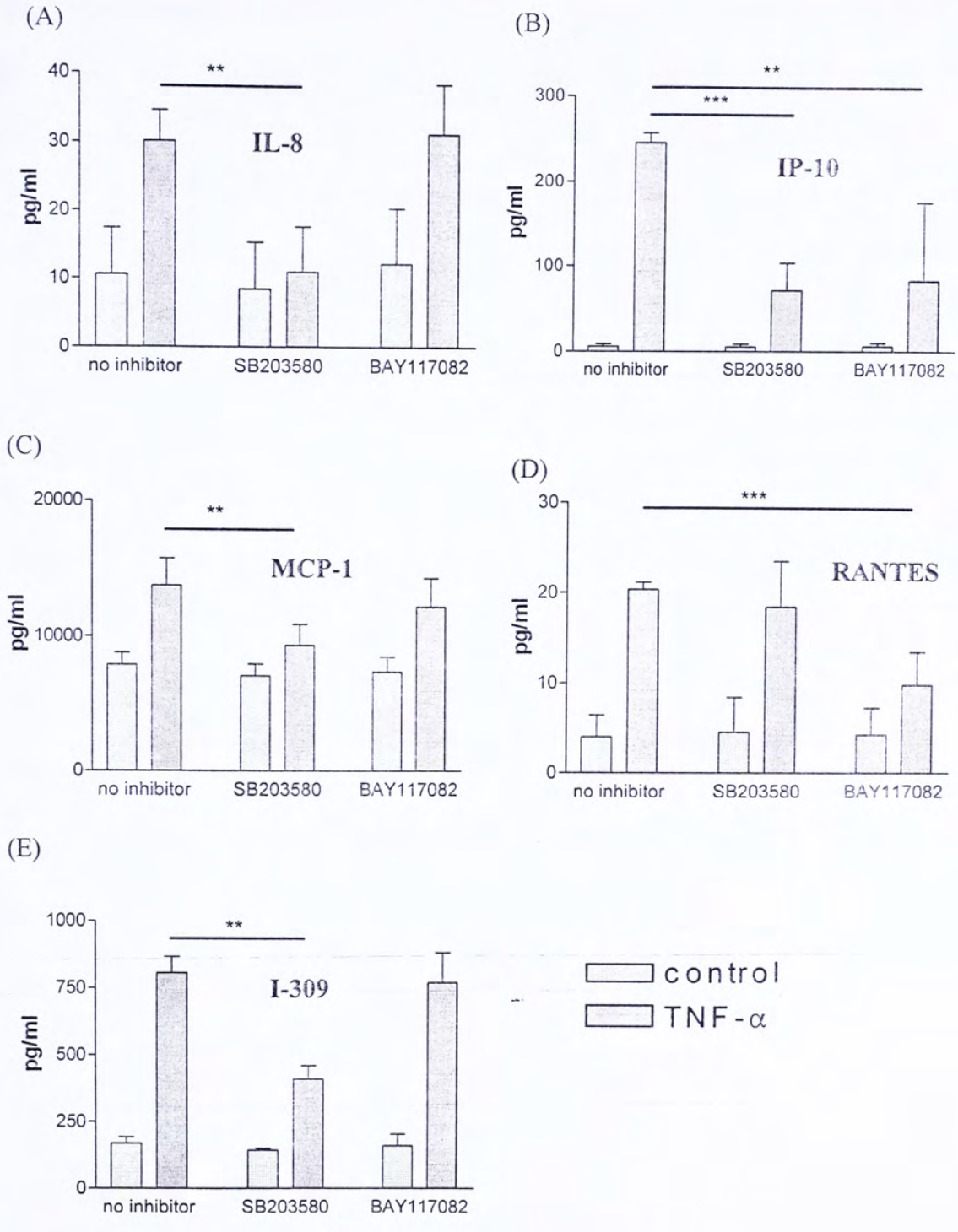

(A)
IL-8
pg/ml
40
30
20
10
0
**
no inhibitor
SB203580
BAY117082
(B)
IP-10
pg/ml
300
200
100
0
**
***
no inhibitor
SB203580
BAY117082
(C)
MCP-1
pg/ml
20000
10000
0
**
no inhibitor
SB203580
BAY117082
(D)
RANTES
pg/ml
30
20
10
0
***
no inhibitor
SB203580
BAY117082
(E)
I-309
pg/ml
1000
750
500
250
0
**
no inhibitor
SB203580
BAY117082
control
TNF-α

Fig. 3.13. Effects of SB203580 and BAY117082 on the TNF-α-induced (A) IL-8, (B) IP-10, (C) MCP-1 (D) RANTES and (E) I-309 in HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated without or with SB203580 (20 μM) BAY117082 (70 μM) for 1 h followed by stimulation with TNF-α for 24 h. The chemokine expression was detected using cytometric bead array and ELISA. Mann-Whitney rank sum test was used to assess the difference between inhibitor-treated group and the non-inhibitor-treated group. Results are expressed as mean plus SD of triplicate experiments. $^{**}p < 0.01$ and $^{***}p < 0.005$.

3.5 The effect of inhibitors of intracellular signal transduction molecules on the SCF and TNF-α-induced upreulation of ICAM-1

3.5.1 BAY117082 but not SB203580 suppressed the TNF-α-induced ICAM-1 expression

As shown in Figure 3.14, BAY117082 could significantly suppress the effect of TNF-α-induced ICAM-1 expression ($p < 0.05$). SB203580 and PD98059 showed no effect on TNF-α-induced ICAM-1 expression on HMC-1 cells ($p > 0.05$).

3.5.2 PD98059 and BAY117082 suppressed the combined treatment of SCF and TNF-α induced ICAM-1 expression

Figure 3.14 shows that both PD98059 and BAY117082 could significantly suppress the combined effect of SCF and TNF-α induced ICAM-1 expression ($p < 0.01$ and $p < 0.05$ respectively). Combined treatment of PD98059 and BAY117082 could synergistically suppress the ICAM-1 expression induced by the combined treatment of SCF and TNF-α ($p < 0.005$). SB203580 showed no effect on any SCF- or TNF-α-induced ICAM-1 expression on HMC-1 cells ($p > 0.05$).

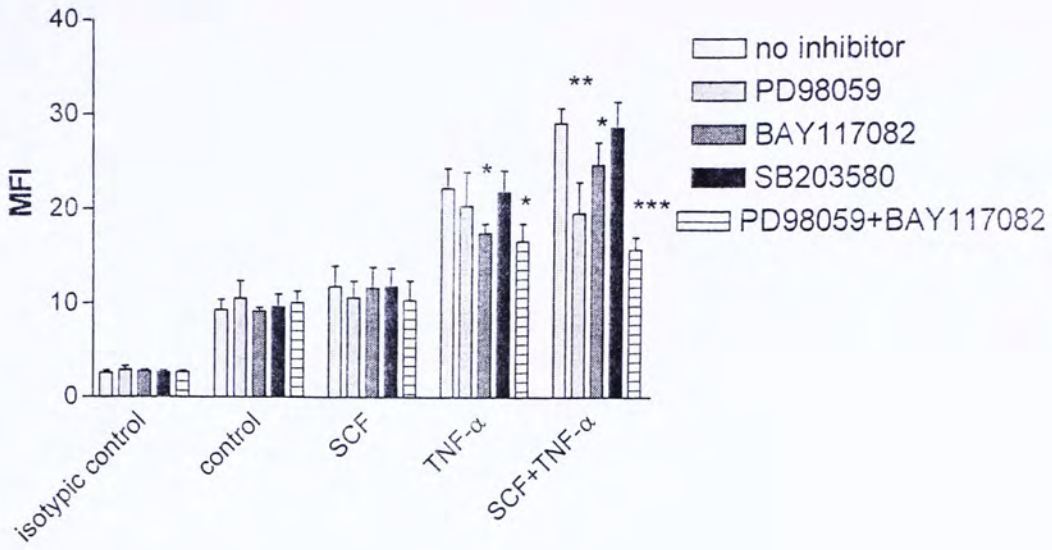


Fig. 3.14. Effects of PD98059, BAY117082 and SB203580 on the SCF- and TNF-α-induced ICAM-1 expression on HMC-1 cells. HMC-1 cells (1 x 10^6 cells/ml) were treated without or with PD98059 (50 μM), BAY117082 (70 μM) and SB203580 (20μM) for 1 h followed by stimulation with SCF, TNF-α and SCF+TNF-α for 48 h. The ICAM-1 expression was detected by flow cytometry using mAb IgG_1 isotype control antibody and mAb against ICAM-1. Results are expressed as MFI and Mann-Whitney rank sum test was used to assess the difference between inhibitor-treated group and the non-inhibitor-treated group under the same cytokine activation. Results are expressed as mean plus SD of 9 experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$.

3.6 Effects of inhibitors on TNF-α and SCF-induced ERK, p38 MAPK and NF-κB activities in HMC-1 cells.

To confirm the inhibitory effects of PD98059, SB203580 and BAY117082 on the activation of ERK, p38 MAPK and NF-κB pathways in HMC-1 cells, the level of phosphorylated ERK, ATF-2 and nuclear NF-κB were determined after the pretreatment of inhibitors.

3.6.1 PD98059 suppressed the SCF-induced activity of ERK

Since PD98059 suppresses the kinase activity of MEK, that is the upstream kinase of ERK, an assay of phosphorylated ERK was performed to confirm the inhibitory effect of PD98059. Pretreatment of ERK inhibitor PD98059 could suppress the SCF-induced phosphorylation of ERK (Figure 3.15A, $p < 0.005$).

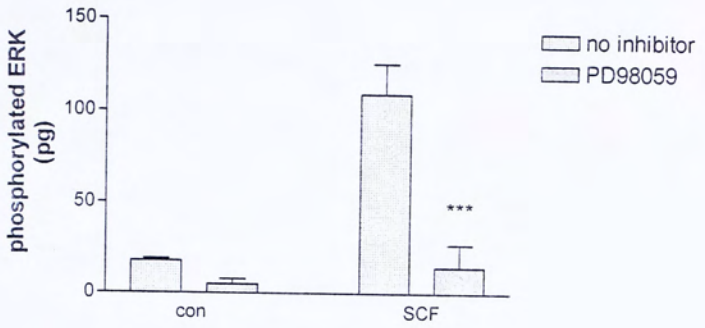
3.6.2 SB203580 and BAY117082 suppressed the TNF-α induced p38 MAPK and NF-κB activity respectively

As SB203580 inhibits the kinase activity of p38 MAPK, the phosphorylation of its downstream substrate ATF-2 can reflect the inhibitory ability of SB203580. Figure 3.15B shows that SB203580 suppressed the TNF-α induced p38 MAPK catalyzed phosphorylation of ATF-2 in HMC-1 cells. BAY117082 inhibits the phosphorylation of IκB and could significantly decrease the TNF-α-induced translocation of NF-κB into the nucleus ($p < 0.001$, Figure 3.15C) in HMC-1 cells.

3.6.3 PD98059 suppressed the enhanced NF-κB activity after the combined treatment of SCF and TNF-α for 18 hours

To illustrate whether the prolonged activation of NF-κB pathway under the combined treatment of SCF and TNF-α could be suppressed by inhibitor of ERK pathway, PD98059 was added before assaying the nuclear translocated NF-κB. The enhanced NF-κB activity upon the combined treatment of SCF and TNF-α for 18 hours could be significantly suppressed by PD98059 ($p < 0.05$, Figure 3.16). No significant effect was found in the shorter period (2 and 7 h) of treatment ($p > 0.05$).

(A)



(B)

Control TNF-α TNF-α SB203580 +SB203580

(C)

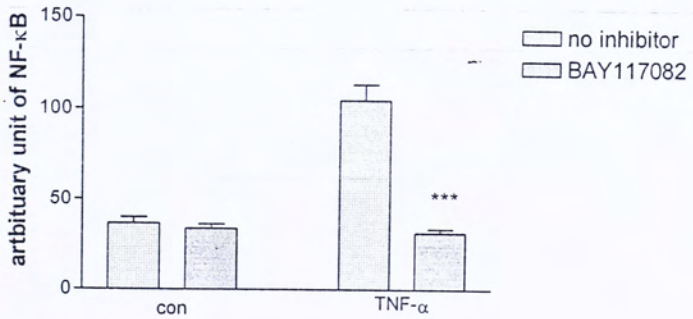


Fig. 3.15. Effects of inhibitors on TNF-α and SCF-induced phosphorylated ERK, p38 MAPK and NF-κB activities in HMC-1 cells. HMC-1 cells (1×10^6 cells/ml) were stimulated by SCF (50 ng/ml) or TNF-α (20 ng/ml) for different time with or without 1 h pretreatment of PD098059 (50 μM), SB203580 (20 μM) or BAY117082 (70 μM). Total cellular proteins were extracted from HMC-1 cells for the measurement of (A) phosphorylated ERK using ELISA at 30 min and (B) p38 MAPK activity by the detection of phosphorylated ATF-2 using p38 MAP Kinase assay kit at 15 min. In Figure (C) nuclear protein was extracted at 7 h for NF-κB protein/NF-κB oligonucleotide binding assay. Experiments were performed in four independent replicates with essentially identical results, and representative results are shown. *** $p < 0.005$.

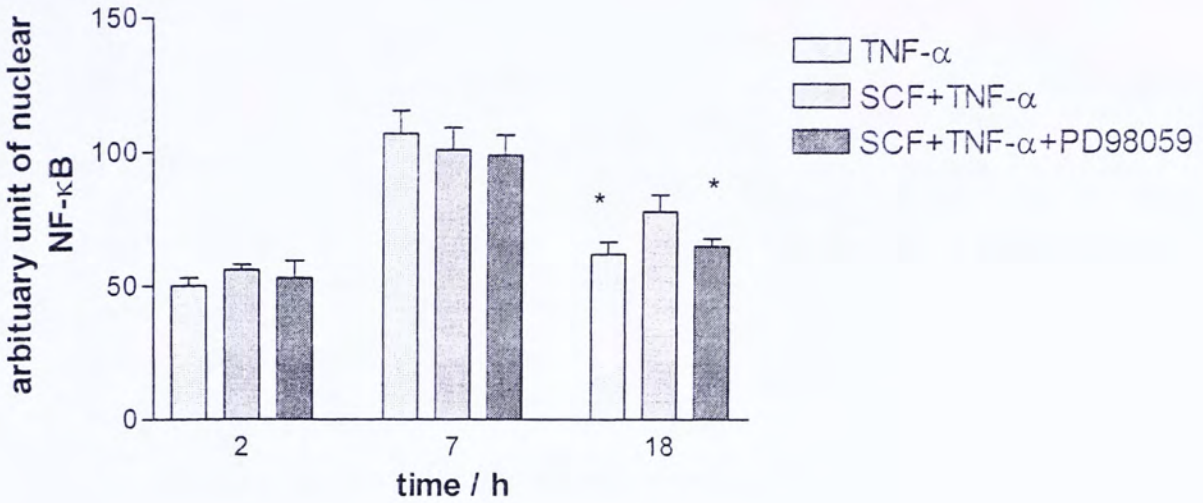


Fig. 3.16. Effects of PD98059, SCF and TNF-α on the NF-κB activities in HMC-1 cells. HMC-1 cells (1 x 10^6 cells/ml) were pre-treated with or without PD98059 (50 μM) for 1 h followed by stimulation with TNF-α and SCF+TNF-α. Nuclear NF-κB protein was extracted after the treatment for 2, 7 and 18 h. Mann-Whitney rank sum test was used to assess the difference between all the treatment within the same time point. Results are expressed as mean plus SD of quadruplicate experiments. * $p < 0.05$.

3.7 Effect of TNF-α and SCF on the gene expression profile of inflammatory cytokine and receptors of HMC-1 cells.

To obtain the gene expression profile of 96 genes related with inflammation, a gene array of inflammatory cytokines/ chemokines and receptors was used. Figure 3.17 shows the hybridization results using cDNA reverse-transcribed from RNA extracted from cells treated without or with SCF and TNF-α.

The 96 genes included in the array can be divided into the following groups:

- Cytokines: IL-10, IL-11, IL-12A, IL-12B, IL-13, IL-15, IL-16, IL-17, IL-18, IL-1A, IL-1B, IL-2, IL-20, IL-25, IL-4, IL-5, IL-6, IL-9, TGF-β, TGF-β1, TGF-β2, TGF-β3, LT-α, LT-β, TNF-α, IFN-γ, LEP, MIF.
- Cytokine receptors: IL-10Rα, IL-10Rβ, IL-11Rα, IL-12Rβ1, IL-12Rβ2, IL-13Rα1, IL-13R, IL-15Rα, IL-17R, IL-18R1, IL-1R1, IL2Rα, IL-2Rβ, IL-2R, IL-5α, IL-6R, IL-6ST, IL-9R, LT-βR, TNF-αR1, TNF-αR2
- Chemokines: I-309, eotaxin, MCP-4, HCC-1, MIP-1δ, HCC-4, TARC, PARC, SCYA19, MCP-1, MIP-3α, MIP-2, MDC, MPIF-1, MPIF-2, TECK, MIP-1α, SCYA4, RANTES, MCP-3, MCP-2, SDF-1, IP-10, I-TAC, SCYB13, ENA-78, GCP-2, lymphotactin, SCM-1β, fractalkine, EMAP-2, SDF-2
- Chemokine receptors: CCR-1, CCR-2, CCR-3, CCR-4, CCR-5, CCR-6, CCR-7, CCR-8, CCR-9, CCXCR1, CX3CR1, CXCR4, CXCR5

The gene list, layout table with gene symbol and position information are given in Appendix I while the results of all detectable genes are shown in Table 3.1. The expression of β-actin (G14, H14) remained constant in both the presence and absence of cytokines. The intensity of β-actin was taken as the positive internal control and given an arbitrary

unit of 100. As shown in Table 3.1, HMC-1 cells basally expressed a wide spectrum of genes of cytokines, chemokines and their receptors; and the expression of all these genes could be differentially affected by the treatment of SCF and TNF-α.

(A) Control (B) SCF (C) TNF-α

(D)

	A	B	C	D	E	F	G	H
1	BLR1	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7
2	CCR8	CCR9	CCXCR1	CX3CR1	CXCR4	IFNG	IL10	IL10R
3	IL10RB	IL11	IL11RA	IL12A	IL12B	IL12RB1	IL12RB2	IL13
4	IL13RA1	IL13RA2	IL15	IL15A	IL16	IL17	IL17R	IL18
5	IL18R	IL1A	IL1B	IL1R1	IL1R2	IL2	IL20	IL21
6	IL25	IL2RA	IL2RB	IL2RG	IL4	IL5	IL5RA	IL6
7	IL6R	IL6ST	IL9	IL9R	LEP	LTA	LTB	LTBR
8	MIF	SCYA1	SCYA11	SCYA13	SCYA14	SCYA15	SCYA16	SCYA17
9	SCYA18	SCYA19	SCYA2	SCYA20	SCYA21	SCYA22	SCYA23	SCYA24
10	SCYA25	SCYA3	SCYA4	SCYA5	SCYA7	SCYA8	SCYB10	SCYB11
11	SCYB13	SCYB5	SCYB6	SCYC1	SCYC2	SCYD1	SCYE1	SDF1
12	SDF2	TGFA	TGFB1	TGFB2	TGFB3	TNF	TNFRSF1A	TNFRSF1B
13	PUC18	PUC18	PUC18	BLANK	BLANK	BLANK	GAPD	GAPD
14	PPIA	PPIA	PPIA	PPIA	RPL13A	RPL13A	ACTB	ACTB

Fig. 3.17. Effects of SCF and TNF-α on the gene expression profiles of inflammatory cytokine and receptor genes. Total RNA was extracted from HMC-1 cells (A) without, and (B) with SCF and (C) TNF-α after 12 h, reverse-transcribed and labeled with biotin, and gene expressions were detected using the GEArray Q series cDNA expression kit. In (D), location of each gene in the array is denoted as symbols correspondingly. The gene located at G14 and H14 (β-actin) were the internal control for each treatment.

Table 3.1. Effects of SCF and TNF-α on the gene expression profile of cytokines, chemokines and their receptor.

			Arbitrary unit of gene expression taking β-actin expression as 100 units		
coordinate	symbol	gene name	control	SCF	TNF-α
cytokine receptors					
3A	IL10RB	IL-10 receptor β	58	85	80
G3	IL12RB2	IL-12 receptor β 2	0	0	12
A4	IL13RA1	IL-13 receptorα 1	45	85	103
G4	IL17R	IL-17 receptor	54	48	60
5A	IL18R1	IL-18 receptor 1	7	22	23
6B	IL2RA	IL-2 receptor	109	151	129
6C	IL2RB	IL-2 receptor	0	14	20
G6	IL5RA	IL-5 receptor	229	214	209
G12	TNFRSF1A	TNF receptor 1	13	23	19
G13	TNFRSF1B	TNF receptor 2	57	94	88
chemokine receptors					
C1	CCR2	CC chemokine recetor 2	11	38	31
F1	CCR5	CC chemokine recetor 5	14	47	55
A2	CCR8	CC chemokine recetor 8	40	80	44
B2	CCR9	CC chemokine recetor 9	0	12	13
D2	CX3CR1	CX3C chemokine recetor 1	135	145	118
cytokines					
C5	IL1B	IL-1β	85	124	160
C7	IL9	IL-9	79	57	127
G2	IL10	IL-10	152	166	174
E4	IL16	IL-16	42	51	54
A6	IL25	IL-25	42	90	44
C12	TGFB1	TGF-β 1	83	92	110
A8	MIF	MIF	0	0	20
chemokines					
B8	SCYA1	I-309	224	209	232
C8	SCYA11	eotaxin	125	133	149
C9	SCYA2	MCP-1	53	84	111
D9	SCYA20	MIP-3α	13	21	31
B10	SCYA3	MIP-1 α	8	22	65
D10	SCYA5	RANTES	13	45	72
D8	SCYB13	BLC	58	66	68
G11	SCYE1	EMAP-2	90	91	90
F9	SCYA22	MDC	0	0	10
G9	SCYA23	MPIF-1	0	0	10
C11	SCYB6	GCP-2	0	8	31
F11	SCYD1	fractalkine	0	26	62
H11	SDF1	SDF-1	0	19	17

β-actin was taken as the internal control for each treatment and as the 100 arbitrary units of gene expression.

3.8 The effects of TCM on the SCF-induced I-309 and MCP-1 from HMC-1 cells

3.8.1 Endotoxin level of *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis*

Endotoxin is referred to the LPS complex associated with the outer membrane of Gram-negative bacteria. Endotoxin can stimulate the biological activities of leukocytes and elicit a wide spectrum of nonspecific pathophysiological reactions such as fever, cachexia, anorexia, shock and even death in whole body [Lake et al, 2004]. Since TCM is not purified or synthetic chemical compound but cultivated plant, endotoxin may be present in the TCM extracts and give false positive results on the HMC-1 cells. The LAL test was used to determine the endotoxin levels (2.2.16). It was found that the endotoxin levels of *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* were 16.0 ± 5.1, 28.3 ± 0.6, 2.1 ± 0.2, 58.4 ± 11.6 and 3.8 ± 1.4 EU/mg respectively (Figure 3.18). To make sure the effects of TCM on HMC-1 cells were not due to LPS, polymyxin B, an antibiotic that can bind to LPS, was added to the TCM extracts to neutralize the effects of LPS. Previously, polymyxin B (5 μg/ml) had been established to be the optimal dose for neutralizing the effects of endotoxin on PBMC without showing any cytotoxicity (data not shown). The same dose was adopted in the experiments on HMC-1 cells.

3.8.2 The effects of TCM on the proliferation rate of HMC-1 cells

In Figure 3.19, it was shown that all 5 TCM hot water extracts could suppress the proliferation rate of HMC-1 cells in high concentration. The minimal concentrations of

Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae and *Cordyceps sinensis* that could significantly suppress the proliferation rate of HMC-1 cells were 1, 0.1, 5, 5, 1 mg/ml respectively (all $*p < 0.05$). As shown in Figure 3.20, polymyxin B did not affect the effects of TCM on the proliferation rate of HMC-1 cells by comparing the results of polymyxin B treated- and non-treated-TCM.

Three concentrations were chosen for each TCM extracts as the doses used in the next experiments. The concentrations are 2, 1 and 0.5; 0.5, 0.2 and 0.1; 5, 2, and 1; 2, 1 and 0.5; and 2, 1 and 0.5 mg/ml for *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* respectively. These concentrations gave 80 ± 20% of proliferation rate when compared to control (Figure 3.19).

3.9.3 The effects of TCM on the SCF-induced release of I-309 form HMC-1 cells

At all concentrations being tested, *Radix astragali, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* could not significantly suppress the SCF-induced release of I-309 form HMC-1 cells (all $p > 0.05$, Figure 3.21A). Only *Radix scutellariae* (0.5, 0.2 and 0.1 mg/ml) could significantly suppress the I-309 release (all $p < 0.05$, Figure 3.21A). Polymyxin B did not enhance or suppress the release of I-309 after treatment of SCF (Figure 3.21B). It also did not alter the TCM effects as the same trend of results were obtained in experiments with or without the addition of polymyxin B.

3.9.4 The effects of TCM on the SCF-induced release of MCP-1 form HMC-1 cells

Cordyceps sinensis (2 mg/ml) could significantly enhance the SCF-induced release of MCP-1 form HMC-1 cells ($p < 0.05$, Figure 3.22A). On the other hand, *Radix scutellariae* (0.5, 0.2 and 0.1 mg/ml) could significantly suppress the MCP-1 release (all $p < 0.05$,

Figure 3.22A). *Radix astragali, Radix stemonae* and *Bulbus Fritillariae cirrhosae* did not show any significantly effect on the release of MCP-1 (all $p < 0.05$, Figure 3.22A). Polymyxin B did not show any significant effect on the chemokine release (Figure 3.22B).

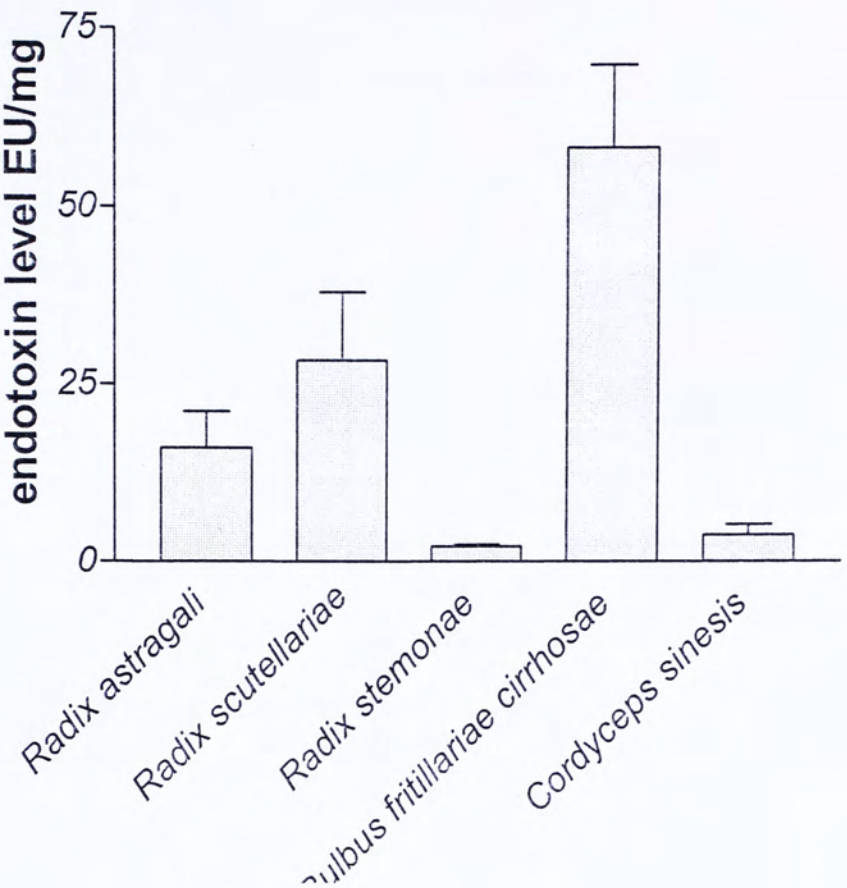
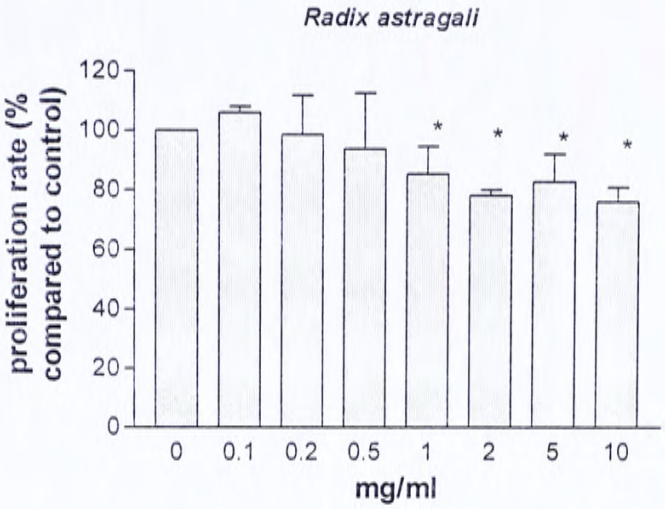
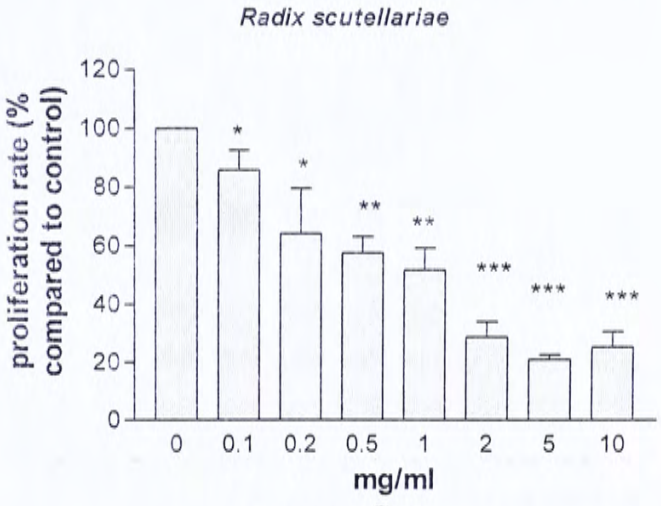


Fig. 3.18 Endotoxin level of TCM. The endotoxin levels of *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* were determined by LAL test and expressed as EU/mg. Results are expressed as mean ± SD of triplicate experiments.

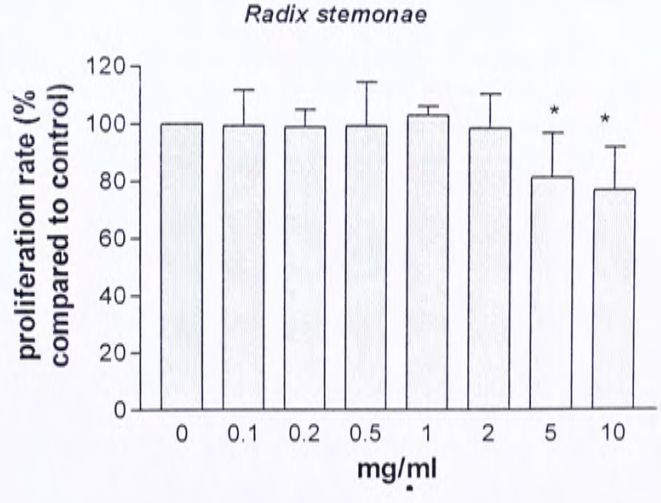
(A)


Radix astragali
proliferation rate (% compared to control)
120
100
80
60
40
20
0
0 0.1 0.2 0.5 1 2 5 10
mg/ml
* * * *

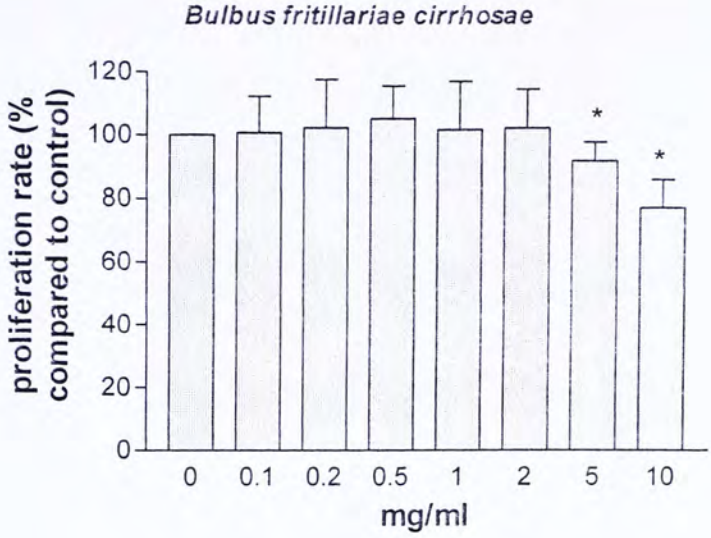
(B)


Radix scutellariae
proliferation rate (% compared to control)
120
100
80
60
40
20
0
0 0.1 0.2 0.5 1 2 5 10
mg/ml
* * ** ** *** *** ***

(C)


Radix stemonae
proliferation rate (% compared to control)
120
100
80
60
40
20
0
0 0.1 0.2 0.5 1 2 5 10
mg/ml
* *

(D)



(E)

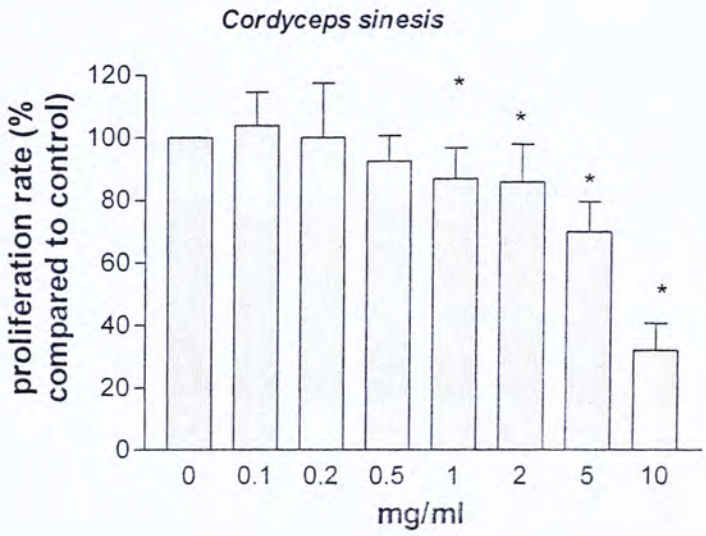
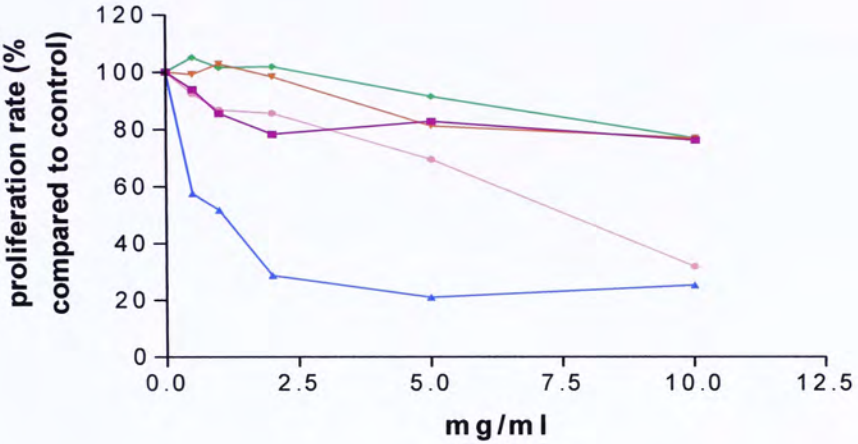
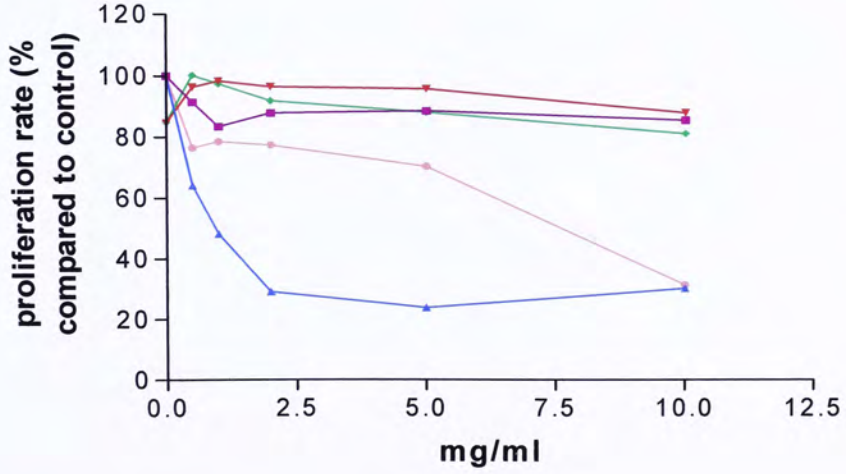


Fig. 3.19 Effects of TCM on the on the proliferation rate of HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated with (A) *Radix astragali, (B) Radix scutellariae, (C) Radix stemonae, (D)Bulbus Fritillariae cirrhosae* and (E) *Cordyceps sinensis* at concentrations of 10, 5, 2, 1, 0.5, 0.2, 0.1 mg/ml for 24 h. All TCM was mixed with polymyxin B (5 μg/ml). The proliferation rate was determined by BrdU ELISA. Percentage of proliferation when compared to control was calculated as: absorbance value of treated cells/ absorbance value of control cells X 100%. Results are expressed as the mean plus SD from four independent experiments. Mann-Whitney rank sum test was used to assess the difference between all treatment groups. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$ when compared with the control.

(A)



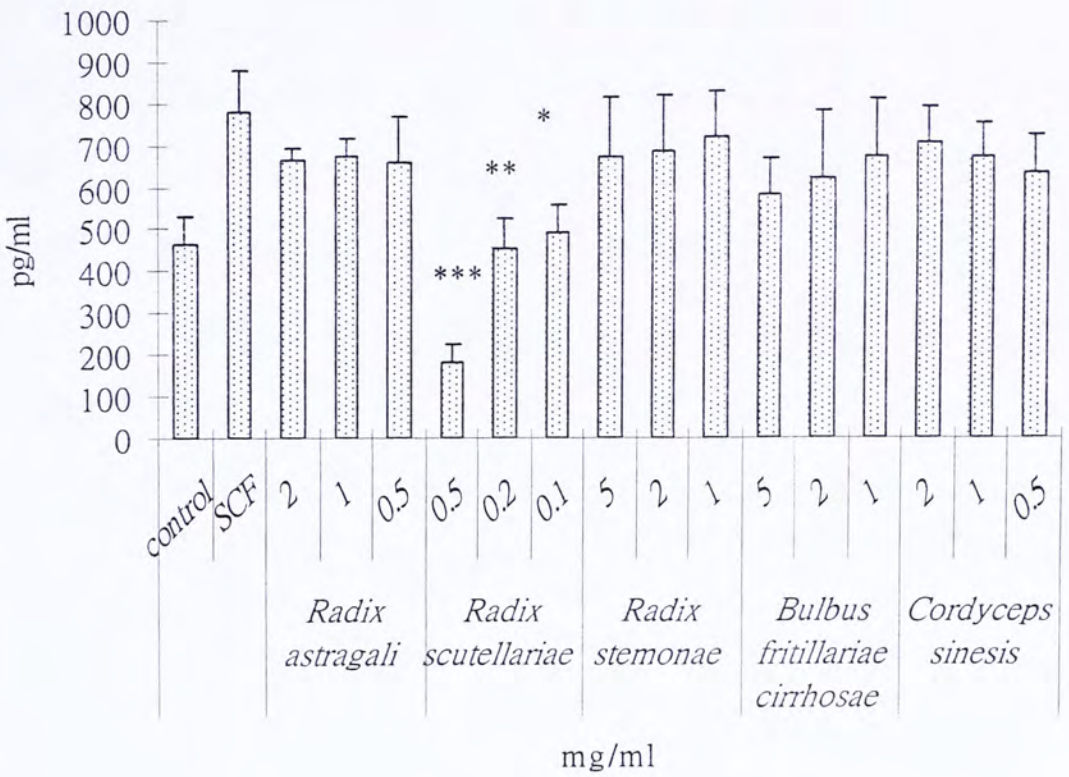
(B)



Radix astragali
Radix scutellariae
Radix stemonae
Bulbus fritillariae cirrhosae
Cordyceps sinesis

Fig. 3.20 Effects of polymyxin B treated- and non-treated-TCM on the proliferation rate of HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated with *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* at concentrations of 10, 5, 2, 1, 0.5 mg/ml for 24 h. TCM was mixed with (A) and without (B) polymyxin B (5 μg/ml). The proliferation rate was determined by BrdU ELISA. Percentage of proliferation when compared to control was calculated as: absorbance value of treated cells/ absorbance value of control cells X 100%.

(A) with polymyxin B



(B) without polymyxin B

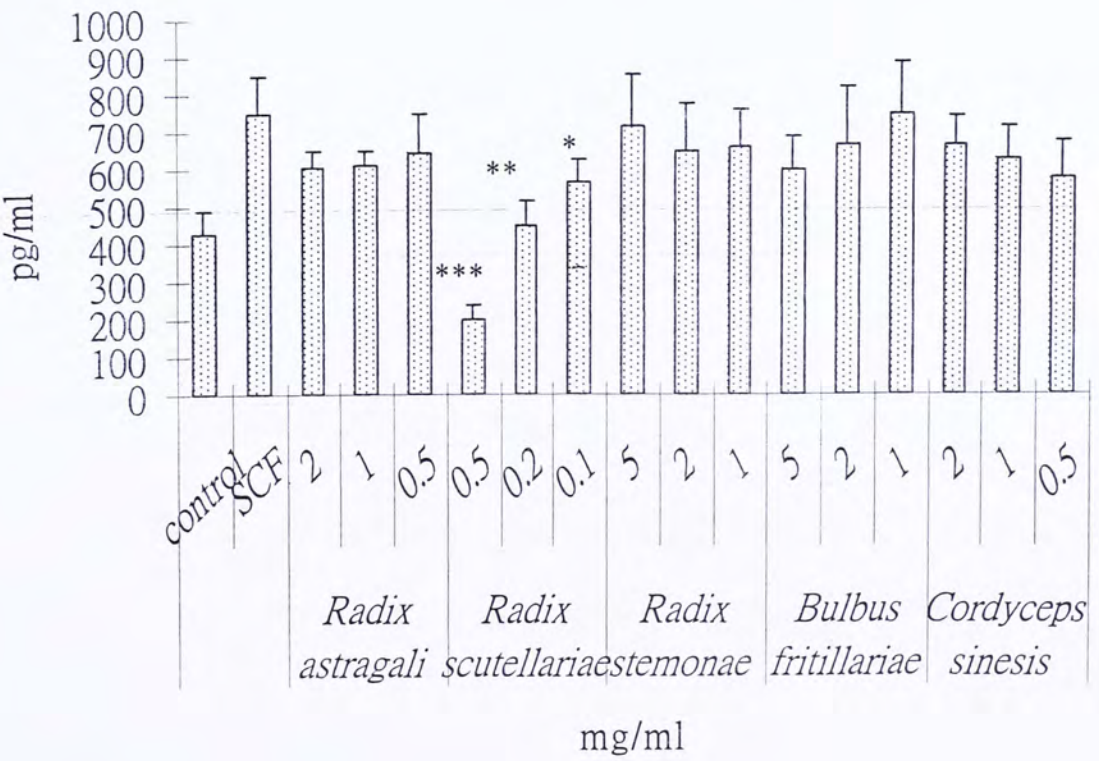
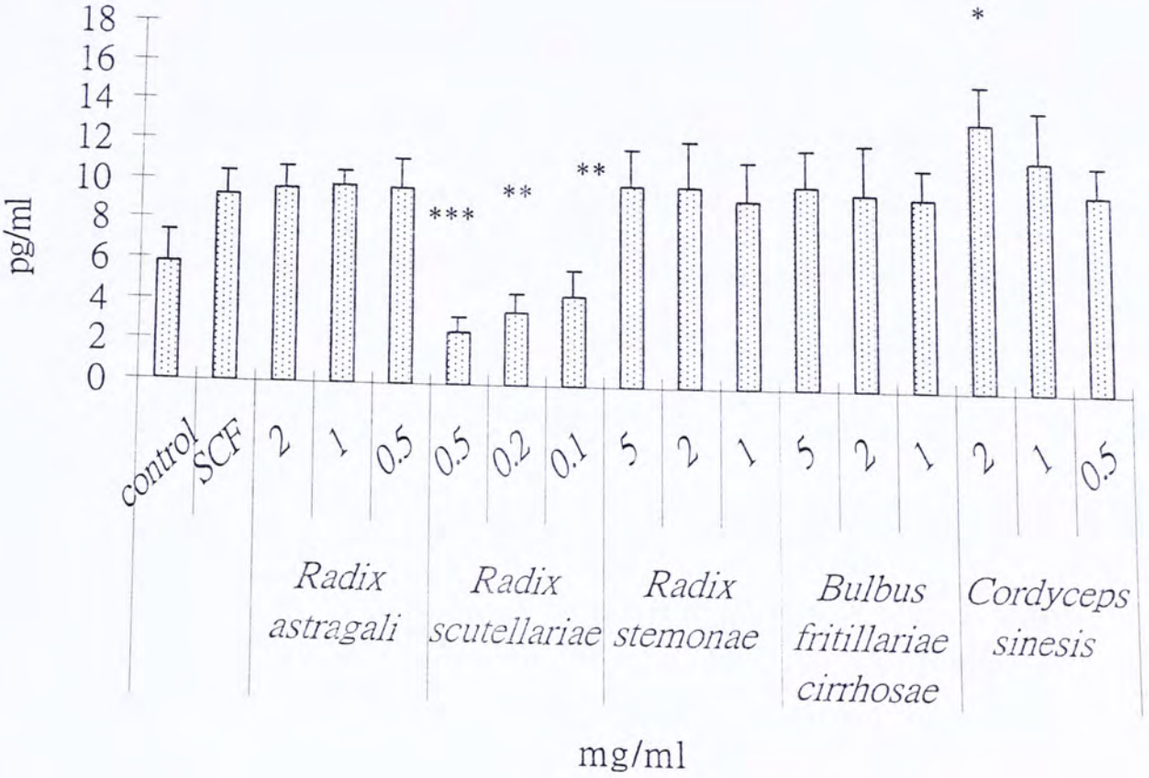


Fig. 3.21 Effects of TCM on the SCF-induced I-309 release from HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated with *Radix astragali* (2, 1 and 0.5 mg/ml), *Radix scutellariae* (0.5, 0.2 and 0.1 mg/ml), *Radix Stemonae* (5, 2 and 1mg/ml) *Bulbus Fritillariae cirrhosae* (5, 2 and 1mg/ml) and *Cordyceps sinensis* (2, 1 and 0.5 mg/ml) for 1 h followed by stimulation with SCF for 24 h. TCM was mixed with (A) and without (B) polymyxin B (5 μg/ml). I-309 released in culture supernatant was detected using ELISA. Mann-Whitney rank sum test was used to assess the difference between (TCM + SCF)-treated group and SCF-treated group. Results are expressed as mean plus SD of four experiments. $*p < 0.05$, $**p < 0.01$ and $***p < 0.005$.

(A)

with polymyxin B



(B)

without polymyxin B

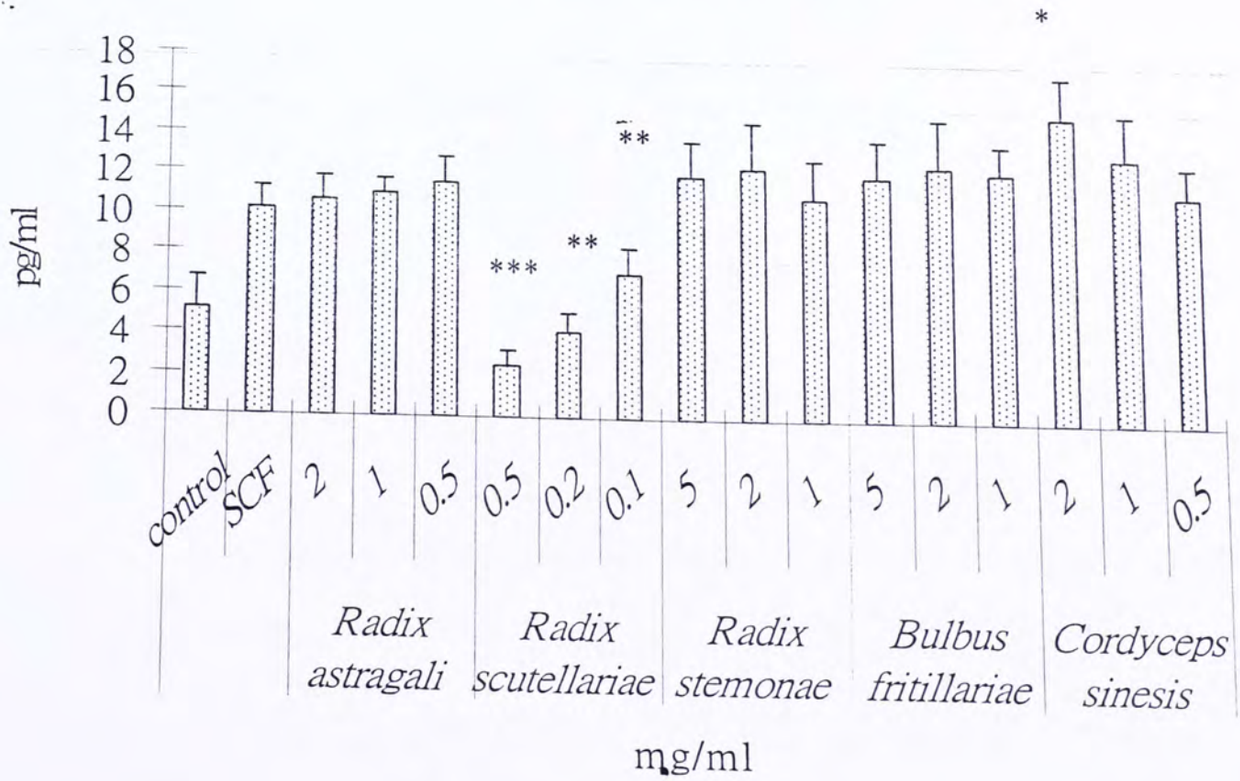


Fig. 3.22 Effects of TCM on the SCF-induced MCP-1 release from HMC-1 cells. HMC-1 cells (2 x 10^6 cells/ml) were treated with *Radix astragali* (2, 1 and 0.5 mg/ml), *Radix scutellariae* (0.5, 0.2 and 0.1 mg/ml), *Radix stemonae* (5, 2 and 1mg/ml) *Bulbus Fritillariae cirrhosae* (5, 2 and 1mg/ml) and *Cordyceps sinensis* (2, 1 and 0.5 mg/ml) for 1 h followed by stimulation with SCF for 24 h. In (A), TCM was mixed with (A) and without (B) polymyxin B (5 μg/ml). MCP-1 released in culture supernatant was detected using ELISA. Mann-Whitney rank sum test was used to assess the difference between (TCM + SCF)-treated group and SCF-treated group. Results are expressed as mean plus SD of four experiments. $^{**}p < 0.01$ and $^{***}p < 0.005$.

Chapter 4
Discussion

4.1 Involvement of adhesion molecules and chemokines in mast cell-mediated immunological events

Mast cells are strategically located along the microvasculature in tissues in close contact with the external environment, such as the skin, lung and intestines. The roles of mast cells in a range of inflammatory and immunological events have been well established [Marone et al, 2005]. For example, mast cells are involved in IgE-mediated mechanisms by cross-linking of surface FcεRI molecules [Okumura et al, 2005]. They also appear to play a key role in defence against bacterial infections [Marshell et al, 2004; Malaviya et al, 2004], and interact with cells of the adaptive immune system [Galli et al, 2005]. All these mast cell-mediated immunological events must be accompanied by accumulation of mast cells. Increase in the local density of mast cells within inflamed tissue have been described in a number of diseases such as asthma [Macfarlane et al, 2000] and rheumatoid arthritis [Ceponis et al, 1998]. The accumulation of mast cells in the inflamed area occurs by recruiting mast cell precursors from the circulation through intercellular interactions of surface adhesion molecules on mast cells. Local maturation of mast cells then takes place and after having stimulated by a range of cytokines in the inflamed tissue, mast cells then become pivotal in mediating leukocyte recruitment to augment the acute and chronic inflammation, mainly through production of chemokines [Selvan et al,1994].

The diverse functions demonstrated by mast cells make us start a project to investigate their roles in inflammation by elucidating the changed expression of

adhesion molecules and chemokines after cytokine stimulations, the intracellular signal transduction governing the responses and also the effects brought by the treatment of anti-allergic TCM.

4.2 HMC-1 as the *in vitro* mast cell model

In the present study, we adopted a human leukemic mast cell line (HMC-1) [Butterfield et al, 1988] as the *in vitro* mast cell model. It is well accepted to serve as an *in vitro* model to study basic mast cell functions and signal transduction [Kempna et al, 2004; Fitzgerald et al, 2004]. In fact, HMC-1 is the only well-established growth factor-independent human mast cell line and has been widely employed for *in vitro* studies of human mast cell biology (Wedi et al, 1996). Another two human mast cell lines, LAD1 and LAD2, established from bone marrow aspirates from a patient with mast cell sarcoma/leukemia [Kirshenbaum et al, 2003], however, are SCF dependent and therefore not suitable for our investigation on cytokine responses.

On the other hand, there are also murine mast cell line MC/9 and rat basophilic leukemia (RBL) mast cell line. Nevertheless, it is known that characteristic of mast cells is species specific and thus both of them are not suitable for the mechanistic study of human diseases [Venkatesha et al, 2005].

Another way to obtain human mast cells is to culture primary cells from umbilical cord blood, bone marrow or peripheral blood. However, it is a time consuming and expensive process that involves the magnetic cell sorting of CD34+ cells followed by culture in the presence of recombinant SCF and IL-6 for at least 15 weeks. In addition, it is noticed that mast cells raised from different tissues show varied responses [Iida et al, 2001].

To start our first project on mast cells, we adopted HMC-1 as our *in vitro* mast cell

model to generate homogenous, robust and reproducible results.

4.3 The effect of cytokines on the ICAM-1 and ICAM-3 expression on HMC-1

Adhesion molecules and their counterreceptors control the immune and inflammatory responses through their specificity and strength of intercellular and matrix interactions [Gonlugur et al, 2004]. They direct the leukocytes from the vascular lumen to migrate into tissue. ICAM-1 and ICAM-3, transmembrane proteins with extracellular domains made up of IgG-like motifs, have been shown to play important role in the recruitment of leukocytes to sites of inflammation and in the mast cell-mediated responses.

ICAM-1 induces allergic responses by mediating mast cell accumulation into inflammatory sites [Wedi et al, 1996]. Profound downregulation of ICAM-1 therefore leads to the elimination of the immediate-type hypersensitivity and the subsequent inhibition of the late phase reaction [Shimada et al, 2003]. The upregulation of ICAM-1 expression contributes to the accumulation of leukocytes [Valent et al, 1991] and facilitates cell-contact-dependent regulation of immune cells in inflamed tissues [Torn et al, 1997].

ICAM-3 is abundantly expressed on mast cells and is suggested to play a key role in adhesive cellular interactions during the initial phase of an immune response [Babina et al, 2002]. Monoclonal antibodies directed against ICAM-3 are capable of inducing rapid HMC-1 cell aggregation and increasing stimulation-dependent release of the pro-inflammatory cytokines IL-6 and IL-8 [Babina et al, 1999].

In the present study, after the stimulation of SCF, TNF-α, IL-13, IL-18 and IL-25, ICAM-3 failed to show an upregulated expression on HMC-1 (Figure 3.1B). However, TNF-α and IL-13 could potently increase the cell surface expression of ICAM-1, while SCF could slightly increase its expression (Figure 3.3).

In general, ICAM-1 on HMC-1 has shown to be more responsive to different cytokines and its upregulated expression has been reported after the treatment of IL-4, TNF-α and IFN-γ [Wedi et al, 1996]. ICAM-3, on the other hand, may play its role in adhesive cellular interactions through its abundant expression (as shown in Figure 3.1B) but not a fluidic expression under the stimulation of cytokines. As reported by Juan M (1999), only the expression of ICAM-1 but not ICAM-3 varied during eosinophil activation, although ICAM-3 is highly expressed in resting eosinophils. Besides, Kessel JM (2003) also reported that the expression of ICAM-3 is not enhanced by proinflammatory cytokines. However, there is another hypothesis suggesting that ICAM-3 on eosinophils retains the cells in agglutinations and downregulation of ICAM-3 can facilitate the migration through endothelium cells. In fact, ICAM-3 down-regulation in eosinophils was recently demonstrated in our laboratory after the treatment of IL-25.

We have also demonstrated the change in expression of ICAM-1 started from the transcriptional level by showing an increase in mRNA quantity after 12 h treatment of SCF, TNF-α and IL-13 [Figure 3.2]. Moreover, the increase in ICAM-1 expression under the stimulation of SCF, TNF-α, IL-13 may also reflect a self-regulatory mechanism in adhesivness of mast cells. It has been proposed by Wedi *et al* that mast cells could self-amplify their adhesive properties via the increased synthesis of ICAM-1 stimulated by TNF-α, IL-4 and IFN-γ [Wedi et al, 1996]. In view of our results regarding the effect of SCF and IL-13 that are also synthesized by mast cells [Gessner et al, 2005], we may conclude that mast cells possess the autocrine function of self-regulating their cell surface expression of ICAM-1.

Besides, this is the first report of an additive effect of SCF and IL-13 and a synergistic effect of SCF and TNF-α on the ICAM-1 cell surface expression (Figure 3.3). We also found that the synergistic effect was dose-dependent on SCF but not TNF-α (Figure 3.4),

suggesting it was mainly due to the effects of SCF. All these results demonstrate cytokines in the inflamed tissue can result in an interactive effect on the expression of ICAM-1 and provide important information to design therapeutics to antagonize the effect of ICAM-1 in inflammatory disorders. As a matter of fact, ICAM-1 has been shown to play a crucial role in mast cell recruitment to the inflamed tissue and interaction with other inflammatory cells [Inamura et al, 1998]. It has also been targeted for treating different inflammatory diseases [Nishibori et al, 2003; Yacyshyn et al, 1999] e.g. Crohn's disease and ulcerative colitis [Van Assche et al, 2002], in which mast cell hyperplasia and degranulation are observed [He et al, 2004]. Antisense ICAM-1 has actually been administered for treating patients with other inflammatory diseases [Nebal et al, 2002]. To further understand the molecular meachanism governing the expression of ICAM-1, the intracellular signal transduction regulating the effect of SCF and TNF-α was investigated and will be discussed in the later part of my project.

4.4 The effect of cytokines on the release of chemokines in HMC-1

Chemokines are small, secreted cytokines that are involved in a variety of immune and inflammatory responses, acting primarily as chemoattractants and activators of specific leukocytes. Chemokines play an essential role in the pathogenesis of mast cells by coordinating trafficking of leukocytes [Bisset et al, 2005]. In view of this, researchers have tried to study the consequent of leukocyte accumulation under the effects of anti-inflammatory compounds, e.g. glucocorticoids on the chemokines generated by mast cells [Schramm et al, 2004]. To further facilitate the understanding of chemokine induction in mast cells, the effects of SCF, TNF-α, IL-13, IL-18 and IL-25 on the release of IL-8, MCP-1, IP-10, RANTES, I-309 and MIP-1β of HMC-1 cells were studied in my project.

We demonstrated that only SCF and TNF-α could upregulate the release of chemokines. SCF could significantly induce IL-8, MCP-1, RANTES, I-309 and MIP-1β while TNF-α could significantly induce IL-8, IP-10, MCP-1, RANTES and I-309 at 24 h incubation from HMC-1 cells (Figure 3.5, all $p < 0.05$). IL-13, IL-18 and IL-25, however, showed no effect of all these chemokines (data not shown). We have also demonstrated that the change in protein level of these chemokines coincided with that of the transcript level (Figure 3.6), suggesting that the regulation of these chemokines is under transcriptional control. We also showed that the SCF and TNF-α did not pose a proliferative effect on HMC-1 cells (Figure 3.7), confirming that the increased released of chemokines was not due to an increase in cell number after treatments.

The potent effect of SCF and TNF-α suggests that these two cytokines are among the most crucial cytokines in eliciting the functions of mast cells. In fact, SCF critically regulates the migration and survival of mast cell precursors, promotes the proliferation of both immature and mature mast cells, enhances mast cell maturation and directly induces secretion of mast cell mediator [Nakahata et al, 2002]. In the presence of SCF, mast cells predominantly produce pro-inflammatory cytokines including IL-1β, IL-6, IL-8, IL-16, and IL-18 [Lorentz et al, 2001]. SCF has been proved to be one of the most important factors influencing mast cell phenotype and function in both health and disease [Galli et al, 1993]. On the other hand, TNF-α is known to be a key inflammatory cytokine and therapeutic target for immunotherapeutic intervention in many chronic inflammatory disorders including rheumatoid arthritis, Crohn's disease and psoriasis [Holtmann et al, 2004]. It also plays a role in the initiation of allergic asthmatic airway inflammation and the generation of airway hyper-reactivity [Thomas , 2001].

Our results clearly illustrate one more role of SCF and TNF-α in mast cell-mediated diseases, i.e. the chemokine inducing property, by showing profound upregulatory effects on IL-8, MCP-1, IP-10, RANTES, I-309 and MIP-1β on HMC-1 cells.

On top of the effect brought out by SCF and TNF-α, we have further confirmed that mast cells can take multi-roles in immunity through the diverse biological actions exhibited by the 6 different chemokines.

First of all, these 6 chemokines are not only chemotactic for either Th1 cells (for cellular immunity) or Th2 cells (for humoral immunity) but both of them. Since distinct T cell subsets express different chemokine receptors, Th1 cells are mostly attracted by RANTES, MIP-1β and IP-10, and Th2 cells are mostly attracted by I-309. MCP-1 and IL-8 are effective on both subsets [Sebastiani et al, 2002]. This illustrates that mast cells can assume different role by attracting Th1 or Th2 cells in different physiological conditions.

Apart from recruiting Th cells, these 6 chemokines are also reported to regulate the trafficking of varied subpopulations of leukocytes in different immunological events after their release from mast cells.

MIP-1β has been shown to profoundly recruit T cells from the circulation to lymph nodes where the primary immune response is initiated [Tedla et al, 1998]. These studies also showed that mast cells are the abundant and major source of MIP-1β in the lymph nodes during the immune response [Wang et al, 1998]. On the other hand, MIP-1β cannot chemoattract B-cells, eosinophils, basophils and mast cells.

I-309, which is constitutively overexpressed by mast cells, is shown to markedly increased after stimulation via high-affinity Fcε receptor I (FcεRI) on mast cells [Nakajima et al, 2002]. Another study reported I-309 chemoattracts Th2 cells to trigger the allergic inflammation [Gilchrest et al, 2003].

MCP-1, which is also constitutively overexpressed in HMC-1, is the crucial chemokine in attracting neutrophils. It has been proposed that peritoneal mast cells are the major producers of MCP-1 responsible for the delayed neutrophil recruitment during acute peritonitis [Wan et al, 2003]. MCP-1 also attracts eosinophils and macrophages in

several inflammatory diseases [Shakoory et al, 2004].

IP-10 is shown to be greatly induced by IFN-α in mast cells during innate reponse against bacterial infection [Mori et al, 2004]. IP-10 appears to specifically target to Th1 cells and shows no effect on neutrophils [Farber, 1997]. Allergic dermatitis and other chronic inflammatory skin diseases have also shown to be mediated by the Th1 cells recruited by IP-10 with mast cells as one of the major sources [Sebastiani et al, 2002].

IL-8 is found to be chemotactic for all known types of migratory immune cells. Reports showed that IL-8 can be induced by SCF and phorbol 12-myristate 13-acetate (PMA) on mast cells and is related with inflammatory diseases like atopic dermatitis and rheumatoid arthritis [Kim et al, 2005]. Another major effect brought out by IL-8 is neutrophil recruitment and degranulation [Park et al, 1998]. Intranasal administration of IL-8 can induce severe granulocytopenia [Van Zee et al, 1992].

It is now clear that mast cells can release a multi-faceted spectrum of chemokines and therefore play a pathophysiological role in numerous inflammatory diseases by coordinating leukocyte trafficking.

Since different cell types are involved in the production of SCF and TNF-α during different inflammatory disorders, the discussion of when and where SCF and TNF-α be produced and their co-relationship with specific diseases are certainly out of the study scope. More clinical investigations are waiting to be carried out in this area and may provide valuable information on understanding the functions of chemokines in mast cell-dependent inflammations for therapeutic intervention.

4.5 The regulation of ICAM-1, IL-8, IP-10, MCP-1, RANTES, I-309 and MIP-1β through p-38 MAPK, ERK and NF-κB signaling pathways in HMC-1

Intracellular signal transduction is a highly interactive network composed of various types of protein kinases and other messenger cascades [Dong et al, 2002; Wong et al, 2004]. Its complexity allows the fine control and integration of the signal transduced that can elicit the precise and diversified cellular responses upon different extracellular stimulations [Pouyssegur et al, 2002]. Since we had demonstrated that SCF and TNF-α had the most significant effect on ICAM-1, IL-8, IP-10, MCP-1, RANTES, I-309 and MIP-1β, we then mainly focused on the effect of SCF and TNF-α on the activation of p38 MAPK, ERK and NK-κB signaling pathways to elucidate the intracellular mechanism in regulating these adhesion molecule and chemokines.

The three pathways were chosen for investigation as they have been mostly correlated with inflammatory diseases by conducting responses for inflammatory cytokines, upregulating adhesion molecules or inducing the release of chemokines to recruit inflammatory cells. It has been shown that p38 MAPK is upregulated by proinflammatory cytokines IL-1β and TNF-α in inflammatory bone disease [Pantouli et al, 2005]. Report also revealed that activity of ERK is significantly higher in asthmatic mice than controls and administration of ERK inhibitor can dramatically inhibit eosinophilia, airway mucus production, and expression of VCAM-1 in lung tissues in the asthmatic mice model [Duan et al, 2004]. The release of chemokines IL-8, IP-10 and MCP-1 are shown to be upregulated through the activation of NK-κB pathways in bronchial epithelial cells co-culturing with eosinophils [Wong et al, 2005].

In the present study, SCF activated ERK while TNF-α activated p38 MAPK and NF-κB pathways (Figure 3.8 – 3.10). SCF-mediated activation of ERK peaked at 30 min and declined afterwards (Figure 3.8). It was also observed that TNF-α did not cause any

phosphorylation of the ERK but caused activation of p38 MAPK reaching peak level at 15 mins and declined afterwards (Figure 3.8 and 3.9). We have also shown that the peak level of nuclear translocated NF-κB protein occurred at 7 hours after treatment of TNF-α or combined treatment of TNF-α and SCF. Afterwards, level of NF-κB protein binding declined, but was found to be higher in the combined treatment of SCF and TNF-α than that of treatment of TNF-α at 18 h (Figure 3.10B).

To illustrate the direct connection of the SCF- and TNF-α-induced upregulation of ICAM-1 and chemokines to the activation of ERK, p38 MAPK and NF-κB pathways, the inhibitors of the corresponding pathways including PD98059, SB203580 and BAY117082 were used. The optimal doses of these inhibitors was found to be 50, 20 and 70 μM respectively that could give the highest inhibitory effect without showing significant cytotoxicity (Figure 3.11).

For the suppressive effect on the release of chemokines, we found that PD98059 (50 μM) could significantly suppress SCF-induced release of IL-8, MCP-1, RANTES, I-309 and MIP-1β from HMC-1 cells (Figure 3.12). On the other hand, SB 203580 (20 μM) could significantly suppress TNF-α-induced IL-8, IP-10, I-309 and MCP-1 while BAY 117082 (70 μM) could suppress IP-10 and RANTES release from HMC-1 cells (Figure 3.13, all $p < 0.05$). With these results, we can summarize that SCF-induced upregulation of IL-8, MCP-1, RANTES, I-309 and MIP-1β are mediated by ERK pathway. And under the treatment of TNF-α, the increased release of IL-8, I-309 and MCP-1 is signaled through the p38 MAPK pathway; RANTES signaled through the NF-κB pathway, and IP-10 signaled through both pathways. Table 4.1 gives a summary of the intracellular signaling regulating the SCF and TNF-α effects on the chemokines.

Table 4.1. Intracellular signaling regulating the SCF and TNF-α effect on the chemokines

Chemokines	SCF-induced upregulation	TNF-α induced upregulation
IL-8	ERK pathway	p38 MAPK pathway
MCP-1	ERK pathway	p38 MAPK pathway
IP-10	SCF did not upregulate its release	p38 MAPK pathway and NF- κB pathway
RANTES	ERK pathway	NF- κB pathway
I-309	ERK pathway	p38 MAPK pathway
MIP-1β	ERK pathway	TNF-α did not upregulate its release

For the inhibitory effect on the ICAM-1 expression, it was shown that BAY117082 but not SB203580 could down-regulate TNF-α-induced ICAM-1 expression on HMC-1 cells (Figure 3.14). It demonstrates that ICAM-1 expression through the NF-κB pathway is independent of the TNF-α-activated p38 MAPK. This result also concurs with the result in our previous report of the TNF-α-induced ICAM-1 expression on eosinophils [IP et al, 2003]. Besides, we also found that PD98059 could suppress the combined treatment of SCF and TNF-α induced synergistic effect of ICAM-1 expression (Figure 3.14) and the SCF-mediated activation of ERK (Figure 3.15A). Morever, the combined addition of PD98059 and BAY117082 could potently inhibit the synergistic upregulation of ICAM-1. Together with the synergistic effect of SCF and TNF-α on ICAM-1 upregulation, we may say SCF acts through the ERK pathway, at least in part, to dose dependently enhance the TNF-α-induced ICAM-1 expression of HMC-1 cells.

In fact, Jiang et al (2002 and 2004) demonstrated a temporal control of NF-κB activation by ERK in rat vascular smooth muscle cells. ERK is shown to enhance the persistent but not the transient activation of NF-κB [Jiang et al, 2004]. In concurrence with these

findings, our results indicated that combined treatment of SCF and TNF-α could prolong the activation of NF-κB when treatment time up to 18 hours (Figure 3.10B), and PD98059 could suppress the enhanced activation (Figure 3.16). On the other hand, there is increasing evidence showing that MAPKs is required for NF-κB-dependent gene expression [Carter et al, 1999] and cross-talk between discrete intracellular signaling pathways [Wong et al, 2004]. As a result, it is quite possible that ERK can crosstalk with the NF-κB upon combined treatment of SCF and TNF-α on ICAM-1 expression to elicit the synergistic effects. However, further experiments are required to lay more evidences on this type of regulatory mechanisms for the discrete response of mast cells.

The reason of studying the intracellular signal transduction in cytokine-mediated responses is due to the anticipation of discovering specific drug that can target a specific component, which is specifically co-related with a disease, in one signaling pathway [Kowalski et al, 2001]. It is obvious that global inhibition of any signaling pathway may result in serious side effect. Despite the fact that p38 MAPK, ERK and NF-κB pathways are all known to be greatly involved in inflammatory diseases, they are also involved in normal cellular physiology or mounting effective immune responses [Wong et al, 2004]. Ways to minimize systemic toxicity are crucial in this aspect of drug intervention. Recently, there are encouraging findings in revealing IKK2 and p65 (components of NF-κB pathway) are associated with rheumatoid-arthritis synoviocytes and inflammatory bowel disease, respectively [Li et al, 2002]. With the increasing knowledge of involvement of signaling pathways in diseases, and advance in computerized programme to discover new small molecules to antagonize activation of different pathways, identifying individual key component for a specific disease will eventually be a new horizon in therapeutics.

In conclusion, the adhesion property and chemokine generation of mast cells are under fine and complicated regulation according to different physiological conditions

and the interactive effect of cytokines. Our results therefore provide insight for cross-talk between different signaling cascades and a better rationale for the design of drug therapy for mast cell-mediated diseases.

4.6 Further characterization of HMC-1 cells using cDNA array

Regarding the study of mast cell interaction with inflammatory cells, we have elucidated the SCF and TNF-α mediated upregulation of ICAM-1, IL-8, MCP-1, IP-10, RANTES, I-309 and MIP-1β. To further explore the interactive roles of mast cells in immunity, we have to target new cytokines as stimulators of HMC-1 and also new chemokines for investigation. Although there are hundreds of publications reporting the expression of cytokines, chemokines and their receptors on mast cells, the information available is fragmented or is some pooled results from all types of mast cells including the human cell line ones, rat cell line ones and primary cell cultured ones.

Consequently, we characterized HMC-1 cells by using an array to simultaneously profile 96 genes associated with inflammatory responses. Despite the facts that gene expression array can only give semi-quantitative results and the gene level may not totally reflects the protein level, gene array is the only array can give information for both cytokines and receptors within one hybridization. In Table 4.2, the results of all detectable gene expression of cytokine and chemokine receptors are listed with their corresponding ligands. Since the gene array only gives semi-quantitative results, the effects of SCF and TNF-α are denoted as 'Yes' for upregulation and 'No' for no effect. We showed that gene expression of receptors IL-10Rβ, IL-13Rα1, IL-17R, IL-18R1, IL2Rα, IL-5Rα, TNF-αR1, TNF-αR2 could be detected without the treatment of SCF and TNF-α, suggesting that the corresponding ligands may be able to stimulate mast cells, and elicit ranges of immune responses. It also implies that IL-10, IL-17, IL-2 and

IL-5 can be served as our new stimulators in our next study.

The gene expression array also gave enormous information for understanding the roles of mast cells in mediating immune cascade by revealing the potential cytokines and chemokines released. The functions of all cytokines and chemokines being expressed in gene level with or without stimulation of SCF and TNF-α are summarized in the Table 4.3 and 4.4. The diverse functions illustrated by the cytokines and chemokines once again confirm the multiple roles of mast cells in immunity.

It is also observed that HMC-1 expressed the mRNA of a great number of chemokines, especially after the stimulation of SCF and TNF-α. All four subfamilies of chemokines including C-C chemokine family (for example, RANTES, MCP-1, MIP-1α, I-309 and eotaxin), the C chemokine family (for example, lymphotactin), C-X-C chemokines (for example, IP-10, GCP-2 and SDF-1) and the C-X_3-C chemokine family (for example, fractalkine), that control different leukocyte recruitment and cellular activation, were shown to be expressed (Table 4.4). Besides, some of the cytokines such as IL-10, IL-16, TGF-β1 and IL-1β also show chemotactic effects on other leukocytes (Table 4.3). These may reflect the recruitment of leukocytes is essentially one of the important roles of mast cells in the inflamed tissues. In fact, mast cells are among the first group of cells arriving the inflammatory sites, it is quite possible that mast cells govern the incoming inflammatory cells by releasing different spectrum of chemokines or cytokines under different stimulations. Within all the chemokines that show mRNA expression in the array, only MCP-1, IP-10, RANTES, I-309 have already been studied (MIP-1β and IL-8 are not included in the array) and therefore all other chemokines may serve as our new targets for investigation.

Table 4.2. The ligands of the cytokine and chemokine receptors being detected in the gene expression array

cytokine receptors	basally expressed	SCF inducible	TNF inducible	ligands	chemokine receptors	basally expressed	SCF inducible	TNF inducible	ligands
IL10RB	Yes	Yes	Yes	IL-10	CCR2	Yes	Yes	Yes	MCP-1, MCP-2, MCP-3, MCP-4
IL12RB2	No	No	Yes	IL-12	CCR5	Yes	Yes	Yes	MIP-1α, MIP-1β,RANTES
IL13RA1	Yes	Yes	Yes	IL-13	CCR8	Yes	Yes	No	I-309
IL17R	Yes	No	Yes	IL-17	CCR9	No	Yes	Yes	TECK
IL18R1	Yes	Yes	Yes	IL-18	CX3CR1	Yes	Yes	No	fractalkine/ neutotactin
IL2RA	Yes	Yes	Yes	IL-2					
IL2RB	No	Yes	Yes	IL-2					
IL5RA	Yes	No	No	IL-5					
IL6ST	Yes	Yes	Yes	IL-6					
TNFRSF1A	Yes	Yes	Yes	TNF					
TNFRSF1B	Yes	Yes	Yes	TNF					

Table 4.3 The general sources and function of the cytokines being detected in the gene expression array.

cytokines	basally expressed	SCF inducible	TNF inducible	general production sources and functions
IL-10	Yes	Yes	Yes	• produced by activated CD8 (+) T-cells, T-helper CD4 (+)T-cell, B-cell lymphomas, monocytes and mast cells [Howard M et al, 1992] • powerful anti-inflammatory cytokine, suppress the production a of IFN-gamma, IL2 and TNF-beta in Th1 T-helper cells [Moore KW et al, 1995] • chemoattract CD8 (+) T-cells [Jinquan T et al, 1993]
IL-16	Yes	Yes	Yes	• produced by lymphocytes, eosinophils, mast cells, fibroblasts and lung epithelium [Cruikshank WW et al, 1994] • chemoattract CD4(+) T-cells, monocytes, eosinophils, and dendritic cells [Kaser A et al, 1999]
IL-1β	Yes	Yes	Yes	• produced by a variety of cells including monocytes, macrophages, neutrophil, endothelial cells, fibroblasts, smooth muscle cells, keratinocytes, Langerhans cells of the skin, epithelial cells, T-cells, B-cells, NK-cells [Beuscher HU et al, 1990] • stimulation T-helper cells to secrete IL2 and to express IL2 receptors • promote B-cells proliferation and the synthesis of immunoglobulins • chemoattract leukocytes especially neutrophils [Schleimer RP et al, 1986]

[Table 4.3 continued]

IL-25	Yes	Yes	No	• produced by CD4 (+) activated memory T cells [Kempuraj D et al, 2003] • Th2 proinflammatory cytokine, induce the production of IL-4, IL-5 and IL-13 [Fort MM et al, 2001] • provoke allergic inflammation especially eosinophil-mediated late phase allergic reactions [Moseley TA et al, 2003]
IL-9	Yes	No	Yes	• produced by mast cells, eosinophils, neutrophils and CD 4(+) T-cells [Petr Panzner et al, 2003] • induce production of mucus and chemokines from pulmonary epithelium, associated with allergic asthma [Longphre M et al, 1999] • enhance eosinophil function via induction of the IL-5 receptor [Gounni AS et al, 2005] • induce immunoglobulin synthesis of all isotypes, especially IgE [Dugas B et al, 1993]
TGF-β1	Yes	No	Yes	• produced by macrophages, lymphocytes, endothelial cells, chondrocytes and leukemia cells [Wang H et al, 2005] • suppress the immune system by inhibiting proliferation of T-cells and B-lymphocytes and cytotoxic activity of natural killer cells [Moses HL et al, 1990] • chemoattract monocytes and neutrophils [Brandes ME., 1991]
MIF	No	No	Yes	• produced by T-cells [Nishihira, J., 2000] • play key role in inflammation and immunity by acting as a cytokine, hormone, or enzyme [Swope MD et al, 1999] • proinflammatory cytokine, stimulate the secretion of TNF-α and IL-1β [Leech M et al, 1999]

Table 4.4. The general sources and function of the chemokines being detected in the gene expression array.

chemokine	basally expressed	SCF inducible	TNF inducible	its receptor	general production sources and functions
I-309	Yes	No	Yes	CCR8 [Haque NS et al, 2001]	• produced by activated monocytes and by T cells [Inngjerdingen M et al, 2000] • chemoattract monocytes and lymphocytes [Miller MD et al, 1992] • favor helper T cell type (Th) 2-associated allergic reactions [D'Ambrosio D et al, 1998]
eotaxin	Yes	Yes	Yes	CCR3 [Ravensberg AJ et al, 2005]	• produced by lymphocytes, macrophages, bronchial smooth muscle cells, endothelial cells and eosinophils [Yamamoto K et al, 2003] • chemoattract eosinophis, mast cells, Th2 type lymphocytes and keratinocytes [Ravensberg AJ et al, 2005] • play a central role in the pathogenesis of allergic airway diseases (asthma and rhinitis), in inflammatory bowel disease and gastrointestinal allergic hypersensitivity [Amerio P et al, 2003]
MCP-1	Yes	Yes	Yes	CCR2 [Rollins, 2001]	• produced by many different cell types including macrophages, neutrophils, fibroblasts, endothelial cells, airway epithelial cells, vascular smooth muscle cells, keratinocytes, and synovial cells etc [Rollins, 2001] • chemoattract monocytes, T-cells, NK-cells, and basophils [Rollins, 2001]

[Table 4.4 continued]

					• polarize naïve T-cells toward Th2 responses when challenged by antigen [Karpus et al, 1997] • mediate inflammatory response by stimulating IL-1 and IL-6 expression from monocytes and histamine release from basophils [Kuna et al, 1992]
MIP-3α	Yes	Yes	Yes	CCR6 [Baba M et al, 1997]	• produced by CD4+ and CD8+ T lymphocytes, B lymphocytes, immature dendritic cells, and activated neutrophils [Yang D et al, 1999] • chemoattract immature dendritic cells, granulocytes and memory T-cells [Homey B et al, 2000] • trigger innate and adaptive immune responses after invasion of enteropathogenic bacteria [Frédéric Sierro et al, 2001]
MIP-1 α	Yes	Yes	Yes	CCR1 [Ramos CD et al, 2005]	• produced by macrophages [Ramos CD et al, 2005] vchemoattract CD8(+) cells [Taub DD et al, 1993] • play important role in acute neutrophilic Inflammation by activating neutrophils, eosinophils, and basophils [Schall TJ et al, 1993]
RANTES	Yes	Yes	Yes	CCR1, CCR4, CCR5 [Zhang et al, 1994]	• produced by circulating T-cells and synovial fibroblasts [Zhang et al, 1994] • chemoattract T-cells, monocytes, eosinophils and basophils [von Hundelshausen et al, 2004]

[Table 4.4 continued]

					• activate eosinophils to release eosinophilic cationic protein and increases the adherence of monocytes to endothelial cells , associating with diseases such as asthma and allergic rhinitis [Lampinen et al, 2004]
BLC	Yes	Yes	Yes	CXCR5 [Schaerli PK et al, 2000]	• produced by stromal cells [Ansel KM et al, 2000] • chemoattract mature B cells and a small subset of T cells [Gunn MD et al, 1998] • recruit B cells to the follicular compartment and its neutralization reduces the severity of collagen-induced arthritis [Zheng B et al, 2005]
EMAP-2	Yes	No	No		• a tumor-derived cytokine [Berger AC et al, 2000] • chemoattract monocyte and granulocytes [Barnett G et al, 2000] • regulating monocyte chemoattraction, endothelial cell activation , and microglial cell reactivity in autoimmune inflammation and embryonic development [Schluesener HJ et al, 1997]
MDC	No	No	Yes	CCR4 [Lezcano-Meza D et al, 2003]	• produced by macrophages and monocyte-derived dendritic cells [Yoshimura T et al, 1987] • chemoattract neutrophilic granulocytes, monocyte-derived dendritic cells and natural killer cells [Godiska et al, 1997] • activate Th2 lymphocytes in the pathogenesis of bronchial asthma [Lezcano-Meza D et al, 2003]

[Table 4.4 continued]

MPIF-1	No	No	Yes	CCR1 [Youn BS et al, 1998]	• produced by macrophages [Patel VP et al, 1997] • chemoattract resting T-cells and monocytes. It is slightly chemotactic for neutrophils [Forssmann U et al, 1997] • potent activator of monocytes and eosinophils [Youn BS et al, 1998]
GCP-2	No	Yes	Yes	CXCR1, CXCR2 [Wuyts A et al, 1997]	• produced by fibroblasts, chondrocytes, endothelial cells and macrophages [Wuyts A et al, 2003] • chemoattract neutrophils [Rovai LE et al, 1997] • activate granulocytes in inflammatory bowel diseases [Gijsbers K et al, 2004]
fractalkine	No	Yes	Yes	CX3CR1 [Liu GY et al, 2005]	• produced by macrophages, endothelial cells and dentritic cells [Imaizumi T et al, 2004] • chemoattract T-cells, natural killer cells and monocytes [Ahn SY et al, 2004] • play an important role in inflammation by acting as both chemoattractant and adhesion molecule [Liu GY et al, 2005]
SDF-1	No	Yes	Yes	CXCR4 [Petit I et al, 2005]	• produced by hematopoietic cells, neuronal, stromal cells, endothelial, and epithelial cells [Aiuti A et al, 1999] • chemoattract T lymphocytes and monocytes [Bleul CC et al, 1996] • play a major role in migration, retention, and development of hematopoietic progenitors in the bone marrow. [Nagasawa T et al, 2000]

4.7 Investigating the *in vitro* anti-allergic activities of a newly developed Wheeze-relief formula using cytokine-activated HMC-1 cells

The study of cytokine-activated HMC-1 cells, not only provided biological and pharmacological implications in Western medicine, but also helped in developing important parameters to investigate the mechanisms of TCM.

TCM is well known to enhance body immunity without showing serious adverse effects. Therefore, it has attracted researchers to demonstrate its use in age-related or chronic diseases. A Wheeze-relief formula composing of 5 herbs of TCM has been developing by the Institute of Chinese Medicine, the Chinese University of Hong Kong, to treat allergic paediatric asthma. Asthma is a chronic and potentially life-threatening pulmonary disease. Its symptoms include coughing, wheezing, shortness of breath and chest tightness. Severe dyspnea, which is due to spasm of bronchial smooth muscles, swelling of the bronchial mucosa, and hypersecretion of the mucus, can be induced upon inhalation of or contact with allergens, such as pollens, house dust mites, insects etc.

Asthma is an old allergic disease and was well documented in ancient books of TCM. Asthma is stated as yin (陰) and qi (氣) deficiencies of the Lung and Kidney. Therefore, the Wheeze-relief formula is composed of the herbs which are known to enter the Lung (入肺經), including *Radix astragali, Radix scutellariae, Radix Stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* [Huang et al, 1999].

In our laboratory, *in vitro* investigation was carried out in the past few years to elucidate the mechanisms of how this formula works. We have shown that the anti-asthmatic effects may be brought out by immunomodulation on eosinophils and PBMC. First of all, the Wheeze-Relief formula could downregulate the expression of chemokine receptor CCR3 on eosinophils. Since CCR3 receptor is one of most important chemokine receptors on eosinophils, the result suggests that the formula can

reduce eosinophils recruitment to the lungs by blocking the chemotactic effects of eotaxin, RANTES, MCP-2, 3 and 4 [Heath et al, 1997]. Secondly, surface expression of CD49d on eosinophils was also reduced after treating with the formula. CD49d has been shown to promote superoxide production from eosinophils through binding with VCAM-1 on endothelial cells [Nagata et al, 1995]. Besides, we have also shown that the eosinophilic cationic protein (ECP) was profoundly inhibited after the drug treatment. As superoxide and ECP are the allergic mediators released by eosinophils [Joseph-Bowen et al, 2004], the Wheeze-Relief formula may help to inhibit the liberation of cytotoxic substance in asthma. Besides, TNF-α, an important mediator in asthma [Ming et al, 1987], was also shown to be downregulated in PBMC after the treatment. It suggests that the formula can reduce the severity of allergic inflammation by suppressing the release of TNF-α.

Since this previous study had mainly focused on demonstrating the effects of the formula in eosinophils and PBMC, mast cells - another effector cell in asthma - was used to further elucidate the effects of the formula. HMC-1 had been shown to increase its release of chemokines after the treatment of SCF (Figure 3.5). Within these chemokines, I-309 and MCP-1 are capable to recruit the Th2 cells that play key roles in bringing out allergic responses [D'Ambrosio et al, 1998 and Rollins, 2001]. Therefore, the effects of the 5 TCM extracts on the released of SCF-induced I-309 and MCP-1 on HMC-1 cells were investigated. Besides, extracts of individual herbs in the formula were used in the investigation instead of using the mixed herbs for treatments as before.

The endotoxin levels of the 5 TCM extracts were firstly assayed. It is because endotoxin may be present in the cultivated herbs and affect the biological activities of leukocytes [Lake et al, 2004]. It was found that each extract of the TCM contained certain amount of endotoxin (Figure 3.18). Consequently, polymyxin B, an antibiotic that can bind to LPS, was added to the TCM extracts so as to neutralize the effects of

LPS and avoid false positive results in our study.

The effects of the 5 TCM extracts on the proliferation rate of the HMC-1 cells were also performed. The aim of doing this experiment was to obtain suitable doses of TCM in the later experiments about the release of I-309 and MCP-1 from HMC-1 cells. The proliferation rate could reflect the general biological functions of the cells. Besides, we could then figure out whether the varied quantity of chemokines was due to a change in cell number or not. *Radix scutellariae* had the most potent suppressive effect on the proliferation even at relatively low concentrations (Figure 3.19). It conincides with the fact that the active ingredient of *Radix scutellariae, baicalin,* can have strong cytotoxic activity against bacteria including staphylococci, cholea, typhoid, *E coli* and pneumococci etc. More importantly, intravenous administration of 27 mg of baicalin can cause a sudden drop in the leukocyte count [Huang et al, 1999]. On the other hand, all TCM extracts could suppress the proliferation of HMC-1 when concentration was high enough (Figure 3.19). It may be mainly due to some non-specific toxic substances in the extracts that can affect the normal function of the cells.

Three concentrations were chosen for each TCM extracts that could retain 80 ± 20% of proliferation rate of HMC-1 cells when compared to control. The concentrations are 2, 1 and 0.5; 0.5, 0.2 and 0.1; 5, 2, and 1; 2, 1 and 0.5; and 2, 1 and 0.5 mg/ml for *Radix astragali, Radix scutellariae, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* respectively (Figure 3.19).

All concentrations of *Radix astragali, Radix stemonae, Bulbus Fritillariae cirrhosae* and *Cordyceps sinensis* could not suppress the release of SCF-induced I-309, showing that these TCM extracts could not inhibit the recruitment of Th2 cells by I-309 released from SCF-activated mast cells (Figure 3.21). *Radix scutellariae* could significantly suppress the release of I-309. However, it was probably a non-specific effect brought out by its high toxicity. It might also be the same case on the release of SCF-induced MCP-1

as *Radix scutellariae* potently suppressed the chemokine release (Figure 3.22).

Radix astragali, Radix stemonae and Bulbus Fritillariae cirrhosae were also not able to downregulate the release of MCP-1 at all concentrations (Figure 3.22). Interestingly, *Cordyceps sinensis* (2 mg/ml) could significantly increase the SCF-induced MCP-1 (Figure 3.22) although it could significantly suppress the proliferation rate of HMC-1 cells at this concentration (Figure 3.19E). It may be explained by the immuno-enhancing property of *Cordyceps sinensis.* In fact, MCP-1, unlike I-309, does not mainly recruit Th2 cells. It also attracts monocytes, neutrophils and NK cells and is shown to be one of the key chemokines in inflammations. Therefore, *Cordyceps sinensis* (2 mg/ml) might activate the HMC-1 cells and enhance the production of MCP-1.

Together, we have not found a potent and specific downregulation of SCF-induced I-309 and MCP-1 by the 5 individual herbs in Wheeze-relief formula. It suggests that the 5 extracts do not exert their anti-allergic effects through suppressing the release of I-309 or MCP-1 from mast cells. The 5 herbs may act on the mast cells in other ways.

On the other hand, the investigation of this formula will eventually progress to doubled blinded, randomized, placebo controlled clinical trials. The Wheeze-relief formula will be in a capsule form consisting of powdered extracts of the five herbs. Children and adolescents aged 5 to 18 years with chronic asthma diagnosed according to criteria proposed by the American Thoracic Society (ATS) (American Thoracic Society, 1987) will be recruited. EDTA-anticoagulated peripheral blood (PB) will be collected for extracting plasma and total RNA of PBMC. Tests for correlating the severity of asthma and prescription of drugs will be performed.

4.8 Concluding remarks and future prospective

From the present study, we have provided important information in deducing biological significance of mast cells in inflammation. A better understanding of the recruitment of mast cells and also their interaction with other inflammatory cells have been facilitated after demonstrating the upregulation of chemokine and adhesion molecule expression in cytokine-activated HMC-1 cells. The investigation of the intracellular signaling pathways also helps in generating pharmacological implications on treating mast cell-mediated diseases.

We have found that ICAM-1, the crucial adhesion molecule in directing mast cells migration from vascular lumen to inflamed tissue, could be upregulated by SCF, TNF-α and IL-13. We have also suggested that mast cells can self-regulate their cell surface expression of ICAM-1 by an autocrine production of cytokines. Besides, an additive effect of SCF and IL-13 and a synergistic effect of SCF and TNF-α on the ICAM-1 cell surface expression were also demonstrated. The study of intracellular signal transduction then revealed that the SCF- and TNF-α-induced ICAM-1 expression on HMC-1 cells was mediated by ERK and NF-κB pathway, respectively. Cross talking of these two pathways was also demonstrated. The results also suggested that the synergistic upregulation of ICAM-1 expression is due to an enhanced activation of NF-κB pathway by the SCF-induced activation of ERK. In this part of study, we found that ICAM-1-mediated mast cell recruitment and adhesion are under fine and complicated regulation according to different physiological conditions and the interactive effect of cytokines. Our results have also provided insight for cross-talk between different signaling cascades and a better rationale for the design of drug therapy for mast cell-mediated diseases.

On the other hand, we have found that SCF and TNF-α had potent effects in

activating mast cells to release the chemokines IL-8, MCP-1, RANTES, IP-10, I-309 and MIP-1β. It suggested that SCF and TNF-α are the key cytokines in the inflamed tissue to mediate the mast cell responses in inflammation. Besides, the results demonstrated the pathophysiological role of mast cells in numerous inflammatory diseases by coordinating leukocyte trafficking through the release of multi-faceted spectrum of chemokines. The understanding of the functions of chemokines may help to generate therapeutic strategies in treating mast cell-mediated diseases. It is because chronic deleterious immune response can be reduced by interfering with the inflammatory actions of chemokines through small molecular inhibitors or antagonists of chemokine receptors or neutralizing antibodies against chemokines or their receptors [Rossi et al, 2000]. Certainly, the study in intracellular signal transduction regulating the chemokine release yet provided more information for therapeutic interventions. We have shown that SCF-induced upregulations of IL-8, MCP-1, RANTES, I-309 and MIP-1β were mediated through ERK pathway. The TNF-α-induced upregulation of IL-8, MCP-1, I-309 and IP-10 were mediated by p38 MAPK pathway. Besides, NF-κB pathway was responsible for the TNF-α-induced IP-10 and RANTES. In fact, the application of intervention of intracellular signal transduction for treating allergic diseases have been investigated using animal model and clinical trials [Duan et al, 2005].

In future, we can further characterize the functions of mast cells in inflammation by using other cytokines as stimulators. As suggested in 4.2, IL-10, IL-17, IL-2 and IL-5 can be used since the receptors for these cytokines have been shown to be expressed on HMC-1 cells. The release of other chemokines such as exotaxin, MIP-3α, MIP-1α, B lymphocyte chemoattractant (BLC), endothelial-monocyte activating polypeptide (EMAP)-2, macrophage derived chemoattractant (MDC), macrophage procoagulant inducing factor (MPIF)-1, granulocyte chemotactic peptide (GCP)-2, fractalkine and stromal cell-derived factor (SDF)-1 after the cytokine stimulation can also be studied as

the mRNA of these chemokines is upregulated by the HMC-1 cells (Table 4.4).

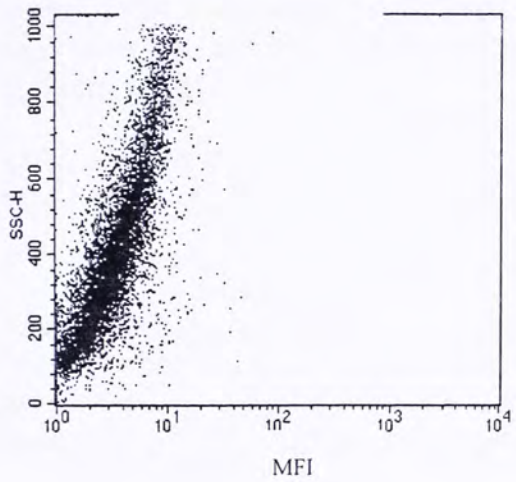
Another way to further study the interaction of mast cells with other inflammatory cells is to assay the expression of cell surface molecules on HMC-1 cells that have been cultured with other cells. The co-culture system can mimic the *in vivo* interaction between different types of cells in the inflammatory site. It is quite possible that the cells will vary their expression of adhesion molecules or receptors after interacting with other cells. Preliminary experiments have been performed on the co-culturing of HMC-1 cells and bronchial epithelial cells (BEAS-2B).When we measure the expression of surface molecules using flow cytometry, we have to firstly gate the two cell populations by using an antibody against SCF receptor (c-kit) that is only present on mast cells. Figure 4.1 shows that the two types of cells could be distinguished by the MFI generated by the antibody against SCF receptor. By using another antibody (conjugated with different fluorescence) against adhesion molecule or receptor, we can easily study the varied expression of any cell surface molecules before and after co-culturing.

Another part of my study was to investigate the *in vitro* anti-allergic activities of the Wheeze-relief formula. However, its five herbal components, including *Cordyceps sinensis*, *Bulbus Fritillariae cirrhosae*, *Radix Stemonae*, *Radix astragali* and *Radix scutellaria* could not show a specific downregulation on the release of SCF- induced I-309 and MCP-1 from HMC-1 cells. It suggests that the five herbs do not exert their anti-allergic effects through suppressing the release of I-309 or MCP-1 from mast cells.

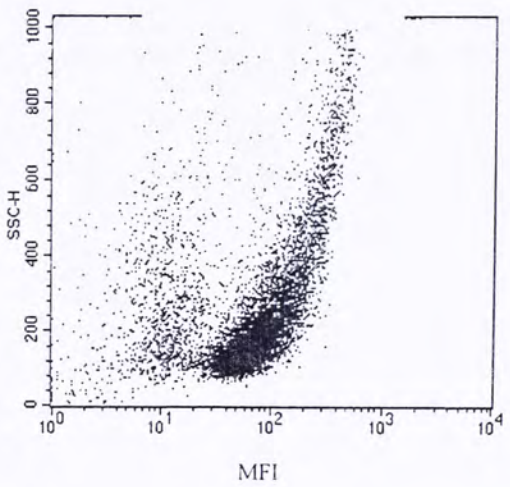
In future, primary cultured mast cells derived from bone marrow, peripheral blood or cord blood could be employed to study the potential therapeutic effects of the formula. It is because primary mast cells express a much higher level of IgE receptors than that of HMC-1 cells. They have been used as a model for investigating the IgE-mediated release of allergic mediators including histamine, leukotrienes and prostagladins [Inamura et al, 1998]. Consequently, the effects of the 5 TCM extracts on the release of

allergic mediators under the IgE challenge could be studied, and the mechanisms of how the formula works on mast cells could also be more clearly elucidated.

(A) BEAS-2B cells



(B) HMC-1 cells



(C) BEAS-2B + HMC-1 cells

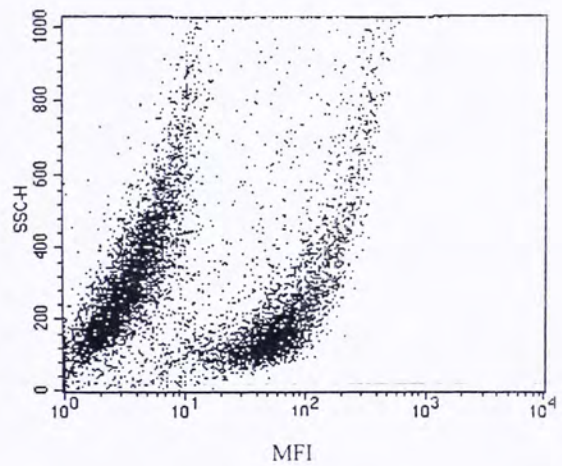


Figure 4.1 The dot plots of HMC-1 cells and BEAS-2B cells using mAb against SCF receptor. HMC-1 cells alone (B) gave much higher MFI than that of BEAS-2B cells (A). In (C), HMC-1 cells and BEAS-2B that have been co-culturing with each other could generate two populations of cells showing different level of MFI.

References

Ahn SY, Cho CH, Park KG, Lee HJ, Lee S, Park SK, Lee IK, Koh GY. 2004. Tumor necrosis factor- induces fractalkine expression preferentially in arterial endothelial cells and mithramycin A suppresses TNF--induced fractalkine expression. Ameri of Pathology. 164: 1663-1672.

Aiuti A. 1999. Human CD34(+) cells express CXCR4 and its ligand stromal cell-derived factor-1. Implications for infection by T-cell tropic human immunodeficiency virus. Blood. 94: 62–73.

Akahira-Azuma M, Szczepanik M, Tsuji RF, Campos RA, Itakura A, Mobini N, McNiff J, Kawikova I, Lu B, Gerard C, Pober JS, Askenase PW. 2004. Early delayed-type hypersensitivity eosinophil infiltrates depend on T helper 2 cytokines and interferon-gamma via CXCR3 chemokines. Immunology. 111: 306-17.

Amerio P, Frezzolini A, Feliciani C, Verdolini R, Teofoli P, De Pita O, Puddu P. 2003. Eotaxins and CCR3 receptor in inflammatory and allergic skin diseases: therapeutical implications. Curr Drug Targets Inflamm Allergy. 2: 81-94.

Ansel KM, Ngo VN, Hyman PL, Luther SA, Förster R, Sedgwick JD, Browning JL, Lipp M, Cyster JG. 2000. A chemokine driven positive feedback loop organizes lymphoid follicles. Nature. 406: 309-314

Baba M, Imai T, Nishimura M. 1997. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. J Biol Chem 272: 14893–14898.

Babina M, Mammeri K, Henz BM. 1999. ICAM-3 (CD50) is expressed by human mast cells: induction of homotypic mast cell aggregation via ICAM-3. Cell Adhes Commun. 7: 195-209.

Babina M, Mammeri K, Henz BM. 2001. Retinoic acid up-regulates myeloid ICAM-3 expression and function in a cell-specific fashion--evidence for retinoid signaling pathways in the mast cell lineage. J Leukoc Biol. 69: 361-372.

Baldwin AS Jr. 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol. 14: 649-683.

Barnett G, Jakobsen AM, Tas M. 2000. Prostate adenocarcinoma cells release the novel proinflammatory polypeptide EMAP-II in response to stress. Cancer Res. 60: 2850-2857

Berger AC, Tang G, Alexander HR, Libutti SK. 2000. Endothelial monocyte-activating polypeptide II, a tumor-derived cytokine that plays an important role in inflammation, apoptosis, and angiogenesis. J Immunother. 23: 519-527.

Berkman N, Robichaud A, Krishnan VL, Roesems G, Robbins R, Jose PJ, Barnes PJ, Chung KF. 1996. Expression of RANTES in human airway epithelial cells: effect of corticosteroids and interleukin-4, -10 and -13. Immunology. 87: 599-603.

Beuscher HU. 1990. IL1beta is secreted by activated murine macrophages as biologically inactive precursor. J Immunol. 144: 2179-2183

Bevilacqua MP. 1993. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol. 11: 767-804.

Bischoff SC, Sellge G. 2002 Mast cell hyperplasia: role of cytokines. Int. Arch. Allergy Immunol. 127: 118-122.

Bisset LR, Schmid-Grendelmeier P. 2005. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. Curr Opin Pulm Med. 11: 35-42.

Blackwell TS, Christman JW. 1997. The role of nuclear factor-kappa B in cytokine gene regulation. Am J Respir Cell Mol Biol. 17: 3-9.

Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med. 184:1101

Bondzi C, Litz J, Dent P, Krystal GW. 2000 Src family kinase activity is required for Kit-mediated mitogen-activated protein (MAP) kinase activation, however loss of functional retinoblastoma protein makes MAP kinase activation unnecessary for growth of small cell lung cancer cells. Cell Growth Differ.11: 305-314.

Brandes ME, Mai UUE, Ohura K, Wahl SM. 1991. Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta. J Immunol. 147: 1600-1606.

Butterfield JH, Weiler D, Dewald G, Gleich GJ. 1988. Establishment of an immature mast cell line from a patient with mast cell leukemia. Leuk. Res. 12: 345-355.

Carter AB, Knudtson KL, Monick MM, Hunninghake GW . 1999 The p38 mitogen-activated protein kinase is required for NF-κB-dependent gene expression. The role of TATA-binding protein (TBP). J Biol Chem. 274: 30858-30863.

Ceponis A, Konttinen YT, Takagi M, Xu JW, Sorsa T, Matucci-Cerinic M, Santavirta S, Bankl HC, Valent P. 1998. Expression of stem cell factor (SCF) and SCF receptor (c-kit) in synovial membrane in arthritis: correlation with synovial mast cell hyperplasia and inflammation. J Rheumatol. 25: 2304-14.

Chialda L, Zhang M, Brune K, Pahl A. 2005. Inhibitors of mitogen-activated protein kinases differentially regulate costimulated T cell cytokine production and mouse airway eosinophilia. Respir Res. 6:36 [Epub ahead of print]

Chung KF, Barnes PJ. 1999. Cytokines in asthma. Thorax. 54: 825-57.

Conti P, Reale M, Barbacane RC, Frydas S, Felaco M, Grilli A, Placido FC, Cataldo I, Feliciani C, Di Gioacchino M, Anogianakis G, Dimitriadou D, Vacalis D, Trakatellis A. 1997. Massive infiltration of basophilic cells in inflamed tissue after injection of RANTES. Immunol Lett. 58:101-6.

Cromwell O, Hamid Q, Corrigan CJ, Barkans J, Meng Q, Collins PD, Kay AB. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. Immunology. 77: 330-7.

Cruikshank WW. 1994. Molecular and functional analysis of a lymphocyte chemoattractant factor: association of biologic function with CD4 expression. PNAS. 91: 5109-5113

Dao T, Ohashi K, Kayano T, Kurimoto M, Okamura H. 1996. Interferon-gamma-inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity of murine T helper 1 cells. Cell Immunol. 173: 230-5.

de Fougerolles A R, Qin X, Springer TA. 1994. Characterization of the function of intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-1 and ICAM-2 in immune responses. *J. Exp. Med. 179:619*

de Groot RP, Coffer PJ, Koenderman L. 1998. Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. Cell Signal. 10: 619-628.

Dinarello CA. 1999. IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol. 103: 11-24.

Dong C, Davis RJ, Flavell RA. 2002 MAP kinases in the immune response. Annu Rev Immunol. 20: 55-72.

Duan W, Chan JH, Wong CH, Leung BP, Wong WS. 2004. Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. J Immunol. 172: 7053-7059.

Duan W, Chan JH, McKay K, Crosby JR, Choo HH, Leung BP, Karras JG, Wong WS. 2005. Inhaled p38alpha mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice. Am J Respir Crit Care Med. 171: 571-578.

Dugas B, Renauld JC, Pene J, Bonnefoy JY, Peti-Frere C, Braquet P, Bousquet J, Van Snick J, Mencia-Huerta JM. 1993. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. Eur J Immunol. 23: 1687-1692

Eblen ST, Slack JK, Weber MJ, Andrew D. 2002. Catling. Rac-PAK Signaling Stimulates Extracellular Signal-Regulated Kinase (ERK) Activation by Regulating Formation of MEK1-ERK Complexes. Molecular and Cellular Biology. 22: 6023-6033

Ernst E. 1998. Complementary therapies in asthma: what patients use. J Asthma 35: 667-671

Farber JM. 1997. Mig and IP-10: CXC chemokines that target lymphocytes. J Leukoc Biol. 61: 246-57.

Fawcett J, Holness CL, Needham LA, Turley H, Gatter KC, Mason DY, Simmons DL. 1992. Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature. 360: 481-484

Feldhaus MJ, Kessel JM, Zimmerman GA, McIntyre TM. 1998. Engagement of ICAM-3 activates polymorphonuclear leukocytes: aggregation without egranulation or β_2 integrin recruitment. J Immunol. 161: 6280-6287.

Fitzgerald SM, Lee SA, Hall HK, Chi DS, Krishnaswamy G. 2004. Human lung fibroblasts express interleukin-6 in response to signaling after mast cell contact. Am J Respir Cell Mol Biol. 30: 585-593.

Forssmann U. 1997. CKbeta8, a novel CC chemokine that predominantly acts on monocytes. FEBS Letters. 408: 211-216

Fort MM, Cheung J, yen D, Li J, Zurawske SM, Lo S, Menon S, Clifford T, Hunte B, Lesley R, Muchamuel T, Hurst SD, Zurawski G, Leach MW, Gorman DM, Rennick DM. 2001. IL-25 induces IL-4, IL-5 and IL-13 and Th2-associated pathologies in vivo. Immunity. 15:985-995

Frédéric Sierro, Bertrand Dubois, Alix Coste, Dominique Kaiserlian, Jean-Pierre Kraehenbuhl and Jean-Claude Sirard. 2001. Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells PNAS. 98: 13722-13727

Frossi B, De Carli M, Pucillo C. 2004. The mast cell: an antenna of the microenvironment that directs the immune response. Leukoc Biol. 75:579-85.

Fukuda M, Gotoh Y, Nishida E. 1997. Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. EMBO J. 16:1901-1908.

Galli SJ, Nakae S, Tsai M. 2005. Mast cells in the development of adaptive immune responses. Nat Immunol. 6:135-42.

Galli SJ, Tsai M, Wershil BK. 1993. The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. Am J Pathol. 142: 965-74.

Gebhardt T, Gerhard R, Bedoui S, Erpenbeck VJ, Hoffmann MW, Manns MP, Bischoff SC. 2005. beta2-Adrenoceptor-mediated suppression of human intestinal mast cell functions is caused by disruption of filamentous actin dynamics. Eur J Immunol. 35: 1124-1132.

Gessner A, Mohrs K, Mohrs M. 2005. Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. J Immunol. 174: 1063-1072.

Gijsbers K, Van Assche G, Joossens S, Struyf S, Proost P, Rutgeerts P, Geboes K, Van Damme J. 2004. CXCR1-binding chemokines in inflammatory bowel diseases: down-regulated IL-8/CXCL8 production by leukocytes in Crohn's disease and selective GCP-2/CXCL6 expression in inflamed intestinal tissue. Eur J Immunol. 34:1992-2000.

Gilchrest H, Cheewatrakoolpong B, Billah M, Egan RW, Anthes JC, Greenfeder S. 2003. Human cord blood-derived mast cells synthesize and release I-309 in response to IgE. Life Sci. 73: 2571-2581.

Godiska R, Chantry D, Raport CJ, Sozzani S, Allavena P, Leviten D, Mantovani A, Gray PW.. 1997. Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. J Exp Med. 185: 1595-1604

Gonlugur U, Efeoglu T. 2004. Vascular adhesion and transendothelial migration of eosinophil leukocytes. Cell Tissue Res. 318: 473-482.

Gottlieb AB, Luster AD, Posnett DN, Carter DM. 1988. Detection of a gamma interferon-induced protein IP-10 in psoriatic plaques. J Exp Med. 168: 941-948.

Gounni AS, Gregory B, Nutku E, Aris F, Latifa K, Minshall E, North J, Tavernier J, Levit R, Nicolaides N, Robinson D, Hamid Q: 2000. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. Blood. 96: 2163-2171

Grabbe J, Welker P, Grutzkau A, Dippel E, Nurnberg W, Zuberbier T, Henz BM. 1998. Induction of human leukaemic mast cell differentiation by fibroblast supernatants, but not by stem cell factor. Scand J Immunol. 47: 324-331.

Grzelewska-Rzymowska I, Pietrzkowicz M. 2004. Role of intra cellular adhesion molecule-1 (ICAM-1) and its soluble form (sICAM) in chronic airway inflammation. Pol Merkuriusz Lek. 16:179-182.

Gunn M D, Ngo VN, Ansel KM, Ekland EH, Cyster JG, Williams LT. 1998. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature 391:799.

Haque NS, Fallon JT, Taubman MB, Harpel PC. 2001. The chemokine receptor CCR8 mediates human endothelial cell chemotaxis induced by I-309 and Kaposi sarcoma herpesvirus-encoded vMIP-I and by lipoprotein(a)-stimulated endothelial cell conditioned medium. Blood. 97:39–45

Hashimoto S, Matsumoto K, Gon Y, Maruoka S, Kujime K, Hayashi S, Takeshita I, Horie T. 2000. p-38 MAP kinase regulates TNF-α-, IL-1α- and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells. Clin Exp Allergy. 30: 48-55.

He SH. 2004. Key role of mast cells and their major secretory products in inflammatory bowel disease. World J Gastro. 10: 309-318.

Heath H, Qin S, Rao P. 1997. Chemokine receptor usage by human eosophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest. 99: 178-184.

Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. 2000. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood. 96: 925-932.

Holtmann MH, Neurath MF. 2004. Differential TNF-signaling in chronic inflammatory disorders. Curr Mol Med. 4: 439-444.

Homey B, Dieu-Nosjean MC, Wiesenborn A. 2000. Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. J Immunol. 164:6621–6632.

Hoshino T, Wiltrout RH, Young HA. 1999. IL-18 is a potent coinducer of IL-13 in NK and T cells: a new potential role for IL-18 in modulating the immune response. J Immunol. 162: 5070-5077.

Howard M. 1992. Biological properties of interleukin 10. J Clin Immunol. 12: 239-47

Huber AR, Kunkel SL, Todd RF, Weiss SJ. 1991. Regulation of transendothelial neutrophil migration by endogenous interleukin-8. Science. 254: 99-102

Huntley A, Ernst E. 2000. Herbal medicines for asthma: a systematic review. Thorax 55: 925-929.

Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, Menon S, Seymour B, Jackson C, Kung TT, Brieland JK, Zurawski SM, Chapman RW, Zurawski G, Coffman RL. 2002. New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. J Immunol. 169: 443-453.

Iida M, Matsumoto K, Tomita H, Nakajima T, Akasawa A, Ohtani NY, Yoshida NL, Matsui K, Nakada A, Sugita Y, Shimizu Y, Wakahara S, Nakao T, Fujii Y, Ra C, Saito H. 2001. Selective down-regulation of high-affinity IgE receptor (FcepsilonRI) alpha-chain messenger RNA among transcriptome in cord blood-derived versus adult peripheral blood-derived cultured human mast cells. Blood. 2001 97: 1016-1022.

Imaizumi T, Yoshida H, Satoh K. 2004. Regulation of CX3CL1/fractalkine expression in endothelial cells. J Atheroscler Thromb. 11:15-21.

Inngjerdingen M, Damaj B, Maghazachi AA. 2000. Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. J Immunol. 164: 4048–4054.

Inamura N, Mekori YA, Bhattacharyya SP, Bianchine PJ, Metcalfe DD. 1998. Induction and enhancement of FcεRI-dependent mast cell degranulation following coculture with activated T cells: dependency on ICAM-1- and leukocyte function-associated antigen (LFA)-1-mediated heterotypic aggregation. J Immunology. 160: 4026-4033.

Ip WK, Wong CK, Lam CWK. 2003. Tumour necrosis factor-α-induced expression of intercellular adhesion molecule-1 on human eosinophilic leukaemia EOL-1 cells is mediated by the activation of nuclear factor-κB pathway. Clin Exp Allergy. 33: 241-248.

Jeffrey DM, Timothy JO, Lauren HM, Jean SM. 2003. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. J Immunol. 170: 1625-1629.

Jelinek DF. 2000. Regulation of B lymphocyte differentiation. Ann Allergy Asthma Immunol. 84: 375-385.

Jiang B, Xu S, Brecher P, Cohen RA. 2002. Growth factors enhance interleukin-1 beta-induced persistent activation of nuclear factor-kappa B in rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 22: 1811-1816.

Jiang B, Xu S, Hou X, Pimentel DR, Brecher P, Cohen RA. 2004. Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. J Biol Chem. 279:1323-1329.

Jinquan T. 1993. Human IL10 is a chemoattractant for CD8 + T lymphocytes and an inhibitor of IL9-induced CD4 + T lymphocyte migration. J Immunol. 151: 4545-4551.

Jo EJ, Lee HY, Lee YN, Kim JI, Kang HK, Park DW, Baek SH, Kwak JY, Bae YS. 2004. Group IB secretory phospholipase A2 stimulates CXC chemokine ligand 8 production via ERK and NF-kappa B in human neutrophils. J Immunol. 173: 6433-6439.

Johns DG, Ao Z, Willette RN, Macphee CH, Douglas SA. 2005. Role of p38 MAP kinase in postcapillary venule leukocyte adhesion induced by ischemia/reperfusion injury. Pharmacol Res. 51: 463-471

Joseph-Bowen J, de Klerk N, Holt PG, Sly PD. 2004. Relationship of asthma, atopy, and bronchial responsiveness to serum eosinophil cationic proteins in early childhood. J Allergy Clin Immunol. 114: 1040-1045.

Juan M, Mullol J, Roca-Ferrer J, Fuentes M, Perez M, Vilardell C, Yague J, Picado C. 1999. Regulation of ICAM-3 and other adhesion molecule expressions on eosinophils in vitro. Effects of dexamethasone. Allergy. 54: 1293-1298.

Julie M. Kessel, Joel Hayflick, Andrew S. Weyrich, Patricia A. Hoffman, Michael Gallatin, Thomas M. McIntyre, Stephen M. Prescott and Guy A. Zimmerman. 1998. Coengagement of ICAM-3 and Fc Receptors Induces Chemokine Secretion and Spreading by Myeloid Leukocytes. J Immunol. 160: 5579-5587.

Kao ST , Lin CS , Hsieh CC , Hsieh WT , Lin JG. 2001. Effects of xiao-qing-long-tang (XQLT) on bronchoconstriction and airway eosinophil infiltration in ovalbumin-sensitized guinea pigs: *in vivo* and *in vitro* studies. Allergy. 56: 1164-1171.

Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. 1997. Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol. 158: 4129-4136.

Kaser A, Dunzendorfer S, Offner FA, Ryan T, Schwabegger A, Cruikshank WW, Wiedermann CJ, Tilg H. 1999. A role for IL-16 in the cross-talk between dendritic cells and T cells. J Immunol. 163:3232.

Kawaguchi M, Adachi M, Oda N, Kokubu F, Huang SK. 2004. IL-17 cytokine family. J Allergy Clin Immunol. 114: 1265-1273.

Kawakami T, Galli SJ. 2002 Regulation of mast-cell and basophil function and survival by IgE. Nat Rev Immunol. 2: 773-786.

Kempna P, Reiter E, Arock M, Azzi A, Zingg JM. 2004. Inhibition of HMC-1 mast cell proliferation by vitamin E: involvement of the protein kinase B pathway. J Biol Chem. 279: 50700-50709.

Kempuraj D, Frydas S, Conti P, Kandere-Grzybowska K, Boucher W, Letourneau R, Madhappan B, Huang SH, Sugimoto K, Papadopoulous NG, Christodoulou S and Theoharides TC. 2003. interleukin -25 (or IL-17E): a new IL-17 family member with growth factor/inflammatory actions. Int J Immunopathol Pharmacol. 16: 185-188

Kernen P, Wymann MP, von Tscharner V, Deranleau DA, Tai PC, Spry CJ, Dahinden CA, Baggiolini M. 1991. Shape changes, exocytosis, and cytosolic free calcium changes in stimulated human eosinophils. J Clin Invest. 87: 2012-2017.

Kessel JM, Gern JE, Vrtis RF, Sedgwick JB, Busse WW. 2003. Ligation of intercellular adhesion molecule 3 inhibits GM-CSF production by human eosinophils. J Allergy Clin Immunol. 111: 1024-1031.

Kim SJ, Lee EJ, Song YS, Jeong HJ, Lee KM, Kim HR, Chae HJ, Shin TY, Kim YK, Hong SH, Kim HM. 2004. Cheongyeolsaseuptang inhibits production of TNF-alpha, IL-6 and IL-8 as well as NF-kappaB activation in human mast cells. J Ethnopharmacol. 97:83-88.

Kimata M, Inagaki N, Kato T, Miura T, Serizawa I, Nagai H. 2000. Roles of mitogen-activated protein kinase pathways for mediator release from human cultured mast cells. Biochem Pharmacol. 60: 589-594.

Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, Rao VK, Metcalfe DD. 2003. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res. 27: 677-682.

KleinJan A, Dijkstra MD, Boks SS, Severijnen LA, Mulder PG, Fokkens WJ. 1999. Increase in IL-8, IL-10, IL-13, and RANTES mRNA levels (in situ hybridization) in the nasal mucosa after nasal allergen provocation. J Allergy Clin Immunol. 103: 441-50.

Kowalski ML, Alam R. 2001. Signal transduction mechanisms as new targets for allergists. Allergy. 56: 199-203.

Kuna P, Reddigari SR, Rucinski D, Oppenheim JJ, Kaplan AP. 1992. Monocyte chemotactic and activating factor is a potent histamine-releasing factor for human basophils. J Exp Med. 175: 489-493.

Kuo YC, Tsai WJ, Wang JY, Chang SC, Lin CY, Shiao MS. 2001. Regulation of bronchoalveolar lavage fluids cell function by the immunomodulatory agents from Cordyceps sinensis. Life Sci. 68: 1067-1082.

Kyriakis JM, Avruch J. 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physio Rev. 81: 808-859.

Lake P, DeLeo J, Cerasoli F, Logdberg L, Weetall M, Handley D. 2004. Pharmacodynamic evaluation of the neutralization of endotoxin by PMX622 in mice. Antimicrob Agents Chemother. 48: 2987-2992.

Lampinen M, Carlson M, Hakansson LD, Venge P. 2004. Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy. 59: 793-805.

Leech M, Metz C, Hall P, Hutchinson P, Gianis K, Smith M, Weedon H, Holdsworth SR, Bucala R, Morand EF. 1999. Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. Arthritis & Rheumatism. 42: 1601–1608.

Leevers S J, Paterson HF, Marshall CJ. 1994. Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature. 369: 411-414.

Leung R, Wong G, Lau J. 1997. Prevalence of asthma and allergy in Hong Kong schoolchildren: an ISAAC study. Eur Respir J. 10: 354-360.

Lezcano-Meza D, Negrete-Garcia MC, Dante-Escobedo M, Teran LM. 2003. The monocyte-derived chemokine is released in the bronchoalveolar lavage fluid of steady-state asthmatics. Allergy. 58: 1125-1130.

Li Q, Verma IM. 2002. NF-κB regulation in the immune system. Nature Rev. 2: 725-734.

Liao JF, Hung WY, Chen CF. 2003. Anxiolytic-like effects of baicalein and baicalin in the Vogel conflict test in mice. Eur J Pharmacol. 464: 141-6.

Liao JF, Shi CC, Chen SY, Fu YT, Chen CF. 1997. Spasmolytic effect of water extract of Stemonae radix on the guinea-pig tracheal smooth muscle in vitro. J Ethnopharmacol. 57: 57-62.

Liu GY, Kulasingam V, Alexander RT, Touret N, Fong AM, Patel DD, Robinson LA. 2005. Recycling of the membrane-anchored chemokine, CX3CL1. J Biol Chem. Mar 16; [Epub ahead of print]

Longphre M, Li D, Gallup M, Drori E, Ordonez CL, Redman T, Wenzel S, Bice DE, Fahy JV, Basbaum C. 1999. Allergen-induced IL-9 directly stimulates mucin in respiratory epithelial cells. J Clin Invest. 104: 1375-1382.

Lorentz A, Bischoff SC. 2001. Regulation of human intestinal mast cells by stem cell factor and IL-4. Immunol Rev. 179: 57-60.

Louahed J, Struyf S, Demoulin JB, Parmentier M, Van Snick J, Van Damme J, Renauld JC. 2003. CR8-dependent activation of the RAS/MAPK pathway mediates anti-apoptotic activity of I-309/ CCL1 and vMIP-I. Eur J Immunol. 33: 494-501.

Luster AD. 1998. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med. 338: 436-445.

Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q, Humbert M, Barnes NC, Robinson DS, Ying S, Kay AB. 2000. Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. J Allergy Clin Immunol. 105: 99-107.

Malaviya R, Ikeda T, Abraham SN, Malaviya R. 2004. Contribution of mast cells to bacterial clearance and their proliferation during experimental cystitis induced by type 1 fimbriated E. coli. Immunol Lett. 91: 103-111.

Marone G, Triggiani M, de Paulis A. 2005. Mast cells and basophils: friends as well as foes in bronchial asthma? Trends Immunol. 26: 25-31.

Marone G, Galli SJ, Kitamura Y. 2002. Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. Trends Immunol. 23: 425-427.

Marshall JS, Jawdat DM. 2004. Mast cells in innate immunity. J Allergy Clin Immunol. 114: 21-27.

Mentink-Kane MM, Wynn TA. 2004. Opposing roles for IL-13 and IL-13 receptor alpha 2 in health and disease. Immunol Rev. 202: 191-202.

Miller MD, Krangel MS. 1992. The human cytokine I-309 is a monocyte chemoattractant. Proc Natl Acad Sci U S A. 89: 2950-2954

Ming WJ, Bersani L, Mantovani A. 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J Immunol. 138: 1469-1474.

Moore KW, Ho ASY, Xu-Amano J. 1995. Molecular biology of interleukin-10 and its receptor. In Interleukin-10. Edited by: DeVries JE and de Waal Malefyt R. , R.G. Landes Company. 1-9.

Mori Y, Hirose K, Suzuki K, Nakajima H, Seto Y, Ikeda K, Shimoda K, Nakayama K, Saito Y, Iwamoto I. 2004. Tyk2 is essential for IFN-alpha-induced gene expression in mast cells. Int Arch Allergy Immunol. 134: 25-29

Moseley TA, Haudenschild DR, Rose L, Reddi AH. 2003. Interleukin-17 family and IL-17 receptors. Cytokine Growth Factor Rev. 14: 155-174

Moser B, Willimann K. 2004. Chemokines: role in inflammation and immune surveillance. Ann Rheum Dis. 63: 1184-1189

Moses HL. 1990. TGF-beta stimulation and inhibition of cell proliferation: new

mechanistic insights. Cell. 63: 245-247

Nagasawa T. 2000. A chemokine, SDF-1/PBSF, and its receptor, CXC chemokine receptor 4, as mediators of hematopoiesis. Int J Hematol. 72: 408–411.

Nagata M, Sedgwick JB, Bates ME, Kita H, Busse WW. 1995. Eosinophil adhesion to vascular cell adhesion molecule-1 activates superoxide anion generation. J immunol. 155: 2194-2202

Nakahata T, Toru H. 2002. Cytokines regulate development of human mast cells from hematopoietic progenitors. Int J Hematol. 75: 350-356.

Nakajima T, Inagaki N, Tanaka H, Tanaka A, Yoshikawa M, Tamari M, Hasegawa K, Matsumoto K, Tachimoto H, Ebisawa M, Tsujimoto G, Matsuda H, Nagai H, Saito H. 2002. Marked increase in CC chemokine gene expression in both human and mouse mast cell transcriptomes following Fcepsilon receptor I cross-linking: an interspecies comparison. Blood. 100: 3861-3868.

Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. 2001. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. Cytokine Growth Factor Rev. 12: 53-72.

Nedbal W, Tomakidi P, Lehmann MJ, Dorfer C, Kohl A, Sczakiel G. 2002. Antisense-mediated inhibition of ICAM-1 expression: a therapeutic strategy against inflammation of human periodontal tissue. Antisense Nucleic Acid Drug Dev. 12: 71-78.

Nishibori M, Takahashi HK, Mori S. 2003. The regulation of ICAM-1 and LFA-1 interaction by autacoids and statins: a novel strategy for controlling inflammation and immune responses. J Pharmacol Sci. 92: 7-12.

Nishihira J. 2000. Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. J. Interferon Cytokine Res. 9: 751–756.

Okumura S, Sagara H, Fukuda T, Saito H, Okayama Y. 2005. FcepsilonRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. J Allergy Clin Immunol. 115: 272-279.

Oliveira SH, Hogaboam CM, Berlin A, Lukacs NW. 2001. SCF-induced airway hyperreactivity is dependent on leukotriene production. Am J Physiol Lung Cell Mol Physiol. 280: 1242-1249.

Oliveira SH, Lukacs NW. 2003. Stem cell factor: a hemopoietic cytokine with important targets in asthma. Curr Drug Targets Inflamm Allergy. 2: 313-318.

Pantouli E, Boehm MM, Koka S. 2005. Inflammatory cytokines activate p38 MAPK to

induce osteoprotegerin synthesis by MG-63 cells. Biochem Biophys Res Commun. 329: 224-229.

Panzner P, Lafitte JJ, Tsicopoulos A, Hamid Q, Tulic MK. 2003. Marked Up-regulation of T Lymphocytes and Expression of Interleukin-9 in Bronchial Biopsies From Patients With Chronic Bronchitis With Obstruction. Chest. 124: 1909-1915.

Papa A, Danese S, Urgesi R, Grillo A, Guglielmo S, Roberto I, Semeraro S, Scaldaferri F, Pola R, Flex A, Fedeli G, Gasbarrini G, Pola P, Gasbarrini A. 2004. Intercellular adhesion molecule 1 gene polymorphisms in inflammatory bowel disease. Eur Rev Med Pharmacol Sci. 8: 187-191.

Park HS, Jung KS, Hwang SC, Nahm DH, Yim HE. 1998. Neutrophil infiltration and release of IL-8 in airway mucosa from subjects with grain dust-induced occupational asthma. Clin Exp Allergy. 28: 724-730.

Patel V P, Kreider B L, Li Y, Li H, Leung K, Salcedo T, Nardelli B, Pippalla V, Gentz S, Thotakura R, Parmelee D, Gentz R, Garotta G. 1997. Molecular and Functional Characterization of Two Novel Human C-C Chemokines as Inhibitors of Two Distinct Classes of Myeloid Progenitors. J Exp Med. 185: 1163-1172

Pawankar R, Yamagishi S, Takizawa R, Yagi T. 2003. Mast cell-IgE-and mast cell-structural cell interactions in allergic airway disease. Curr Drug Targets Inflamm Allergy. 2: 303-312.

Petering H, Gotze O, Kimmig D, Smolarski R, Kapp A, Elsner J. 1999. The biologic role of interleukin-8: functional analysis and expression of CXCR1 and CXCR2 on human eosinophils. Blood. 93: 694-702.

Petit I, Goichberg P, Spiegel A, Peled A, Brodie C, Seger R, Nagler A, Alon R, Lapidot T. 2005. Atypical PKC-ζ regulates SDF-1–mediated migration and development of human CD34+ progenitor cells. J Clin Invest. 115: 168–176.

Pomerantz JL, Baltimore D. 2002. Two pathways to NF-kB. Molecular cell. 10: 693-701.

Pouyssegur J, Volmat V, Lenorman P.2002. Fidelity and spatio-temporal control in MAP kinase (ERKs) signaling. Biochem Pharma. 64: 755-763.

Ramos CD, Canetti C, Souto JT, Silva JS, Hogaboam CM, Ferreira SH, Cunha FQ. 2005. MIP-1{alpha}[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-{alpha} and LTB4. J Leukoc Biol. Apr 14; [Epub ahead of print]

Ravensberg AJ, Ricciardolo FL, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS,

Mauad T. 2005. Eotaxin-2 and eotaxin-3 expression is associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. J Allergy Clin Immunol. 115: 779-785.

Roebuck KA, Finnegan A. 1999. Regulation of intercellular adhesion molecule-1 (CD54) gene expression. J Leukoc Biol. 66: 876-888.

Rollins BJ. 2001. MCP-1, MCP-2, MCP-3, MCP-4, and MCP-5. In: Joost J, Oppenheim, Marc Feldmann, eds. Cytokine reference volume 1: a compendium of cytokines and other mediators of host defense. London; San Diego: Academic Press. 1145 - 1160.

Rottem M., Okada T. Goff J.P. 1994. Mast cells cultured from the peripheral blood of normal donors and patients with mastocytosis originate from a CD34+/Fc0R1- cell population. Blood. 84: 2489-2496.

Roux PP, Blenis J. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 68: 320-344.

Rovai, LE, Herschman, HR, Smith, JB. 1997. Cloning and characterization of the human granulocyte chemotactic protein-2 gene J Immunol. 158: 5257-5266

Ruckes T, Saul D, Van Snick J, Hermine O, Grassmann R. 2001. Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309. Blood. 98: 1150-1159.

Saklatvala J. 2004. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol. 4: 372-377.

Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. 2000. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med. 192:1553.

Schall T J, Bacon K, Camp RD, Kaspari JW, Goedell DV. 1993. Human macrophage inflammatory protein-1 (MIP-1) and MIP-1ß chemokines attract distinct populations of lymphocytes. J Exp Med. 177:1821.

Schleimer RP, Rutledge BK. 1986. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. J Immunol. 136: 649-654.

Schluesener HJ. Localization of endothelial-monocyte-activating polypeptide II (EMAP-2), a novel proinflammatory cytokine, to lesions of experimental autoimmune encephalomyelitis, neuritis and uveitis: expression by monocytes and activated microglial cells. Glia. 20: 365-372

Schramm R, Thorlacius H. 2004. Neutrophil recruitment in mast cell-dependent

inflammation: inhibitory mechanisms of glucocorticoids. Inflamm Res. 53: 644-652.

Sebastiani S, Albanesi C, De PO, Puddu P, Cavani A, Girolomoni G. 2002. The role of chemokines in allergic contact dermatitis. Arch Dermatol Res. 293: 552-559.

Selvan RS, Butterfield JH, Krangel MS. 1994. Expression of multiple chemokine genes by a human mast cell leukemia. J Biol Chem. 269: 13893-13898

Shakoory B, Fitzgerald SM, Lee SA, Chi DS, Krishnaswamy G. 2004. The role of human mast cell-derived cytokines in eosinophil biology. J Interferon Cytokine Res. 24: 271-281.

Shimada Y, Hasegawa M, Kaburagi Y, Hamaguchi Y, Komura K, Saito E, Takehara K, Steeber DA, Tedder TF, Sato S. 2003. L-selectin or ICAM-1 deficiency reduces an immediate-type hypersensitivity response by preventing mast cell recruitment in repeated elicitation of contact hypersensitivity. J Immunol. 170: 4325-4334.

Shon YH, Kim JH, Nam KS. 2002. Effect of Astragali radix extract on lipopolysaccharide-induced inflammation in human amnion. Biol Pharm Bull. 25:77-80.

Staunton DE, Marlin SD, Stratowa C, Dustin ML, Springer TA. 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell. 52: 925-933.

Sundstrom M, Vliagoftis H, Karlberg P, Butterfield JH, Nilsson K, Metcalfe DD, Nilsson G. 2003. Functional and phenotypic studies of two variants of a human mast cell line with a distinct set of mutations in the c-kit proto-oncogene. Immunology. 108: 89-97.

Suzuki T, Takano I, Nagai F, Fujitani T, Ushiyama K, Okubo T, Seto T, Ikeda S, Kano I. 1999. Suppressive effects of Hochu-ekki-to, a traditional Chinese medicine, on IgE production and histamine release in mice immunized with ovalbumin. Biol Pharm Bull. 22: 1180-1184.

Swope MD, Lolis E. 1999. Macrophage migration inhibitory factor: cytokine, hormone or enzyme? Rev Physiol Biochem Pharmacol. 139: 1–32

Tanaka Y. 1993. T cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta Nature (London). 361: 79-82

Tas SW, Remans PH, Reedquist KA, Tak PP. 2005. Signal transduction pathways and transcription factors as therapeutic targets in inflammatory disease: towards innovative antirheumatic therapy Curr Pharm Des. 11: 581-611.

Taub DD, Turcovski-Corrales SM, Key ML, Longo DL, Murphy WJ. 1996. Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte

activation in vitro. J Immunol. 156: 2095-2103.

Taub DD, Conlon K, Lloyd AR, Oppenheim JJ, Kelvin DJ. 1993. Preferential migration of activated CD4 and CD8 T cells in response to MIP-1 and MIP-1ß. Science. 260: 355-358.

Tedla N, Wang HW, McNeil HP, Di Girolamo N, Hampartzoumian T, Wakefield D, Lloyd A. 1998. Regulation of T lymphocyte trafficking into lymph nodes during an immune response by the chemokines macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta. J Immunol. 161: 5663-5672.

Thomas PS. 2001. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol Cell Biol. 79: 132-140.

Torn H, Kinashe T, Ra C, Nonoyama S, Yata J, Nakahata T. 1997. Interleukin-4 induces homotypic aggregation of human mast cells by promoting LFA/ICAM-1 adhesion on molecules. Blood. 89: 3296-3302.

Underwood DC, Osborn RR, Kotzer CJ, Adams JL, Lee JC, Webb EF, Carpenter DC, Bochnowicz S, Thomas HC, Hay DW, Griswold DE. 2000. SB239063, a potent p38 MAP kinase inhibitor, reduces inflammatory cytokine production, airways eosinophil infiltration, and persistence. J Pharmacol Exp Ther. 293: 281-288.

Vaddi K, Newton RC. 1994. Regulation of monocyte integrin expression by beta-family chemokines. J Immunol. 153: 4721-4732.

Valent P, Bevec D, Maurer D, Besemer J, Di Padova F, Butterfield JH, Speiser W, Majdic O, Lechner K, Bettelheim P. 1991. Interleukin 4 promotes expression of mast cell ICAM-1 antigen. Proc Natl Acad Sci USA. 88: 3339-3342.

Van Assche G, Rutgeerts P. 2002. Antiadhesion molecule therapy in inflammatory bowel disease. Inflamm Bowel Dis. 8: 291-300.

Van de Stolpe A, van der Saag PT. 1996. Intercellular adhesion molecule-1. J Mol Med. 74: 13-33.

Van Zee KJ, Fischer E, Hawes AS, Hebert CA, Terrell TG, Baker JB, Lowry SF, Moldawer LL. 1992. Effects of intravenous IL-8 administration in nonhuman primates. J Immunol. 148: 1746-1752.

Venkatesha RT, Berla Thangam E, Zaidi AK, Ali H. 2005. Distinct regulation of C3a-induced MCP-1/CCL2 and RANTES/CCL5 production in human mast cells by extracellular signal regulated kinase and PI3 kinase. Mol Immunol. 42: 581-587.

von Hundelshausen P, Koenen RR, Sack M, Mause SF, Adriaens W, Proudfoot AE, Hackeng TM, Weber C. 2005. Heterophilic interactions of platelet factor 4 and RANTES

promote monocyte arrest on endothelium. Blood. 105: 924-930.

Walsh LJ, Trinchieri G, Waldorf HA, Whitaker D, Murphy GF. 1991. Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1. Proc Natl Acad Sci USA. 88: 4220-4224.

Wan MX, Wang Y, Liu Q, Schramm R, Thorlacius H. 2003. CC chemokines induce P-selectin-dependent neutrophil rolling and recruitment in vivo: intermediary role of mast cells. Br J Pharmacol. 138: 698-706.

Wang H, Mengsteab S, Tag CG, Gao CF, Hellerbrand C, Lammert F, Gressner AM, Weiskirchen R. 2005. Transforming growth factor-beta1 gene polymorphisms are associated with progression of liver fibrosis in Caucasians with chronic hepatitis C infection. World J Gastroenterol. 11: 1929-1936.

Wang HW, Tedla N, Lloyd AR, Wakefield D, McNeil PH. 1998. Mast cell activation and migration to lymph nodes during induction of an immune response in mice. J Clin Invest. 102: 1617-1626.

Wedi B, Elsner J, Czech W, Butterfield JH, Kapp A. 1996. Modulation of intercellular adhesion molecule 1 (ICAM-1) expression on the human mast-cell line (HMC)-1 by inflammatory mediators. Allergy. 51: 676-684.

Welker P, Grabbe J, Zuberbier T, Grutzkau A, Henz BM. 2001. GM-CSF downmodulates c-kit, Fc(epsilon)RI(alpha) and GM-CSF receptor expression as well as histamine and tryptase levels in cultured human mast cells. Arch Dermatol Res. 293: 249-258.

Wild JS, Sigounas A, Sur N, Siddiqui MS, Alam R, Kurimoto M, Sur S. 2000. IFN-gamma-inducing factor (IL-18) increases allergic sensitization, serum IgE, Th2 cytokines, and airway eosinophilia in a mouse model of allergic asthma. J Immunol. 164: 2701-2710.

Wills-Karp M. 2004. Interleukin-13 in asthma pathogenesis. Immunol Rev. 202: 175-190.

Wong CK, Cheung PF, Ip WK, Lam CW. 2005. IL-25 induced hemokines and IL-6 release from eosinophils is mediated by p38 MAPK, JNK and NF-{kappa}B. Am J Respir Cell Mol Biol. [Epub ahead of print]

Wong CK, Ip WK, Lam CWK. 2003. Interleukin-3, -5, and granulocyte macrophage colony-stimulating factor-induced adhesion molecule expression on eosinophils by p38 mitogen-activated protein kinase and nuclear factor-[kappa] B. Am J Respir Cell Mol Biol. 29: 133-147.

Wong CK, Ip WK, Lam CWK. 2004. Biochemical assessment of intracellular signal transduction pathways in eosinophils: implications for pharmacotherapy. Crit Rev Clin

Lab Sci. 41: 79-113.

Wong CK, Wang CB, Ip WK, Tian YP, Lam CW. 2005. Role of p38 MAPK and NF-kB for chemokine release in coculture of human eosinophils and bronchial epithelial cells. Clin Exp Immunol. 139: 90-100.

Woolley DE. 2003. The mast cell in inflammatory arthritis. N Engl J Med. 348: 1709-1711.

Wuyts A. 1997. Characterization of synthetic human granulocyte chemotactic protein 2: usage of chemokine receptors CXCR1 and CXCR2 and in vivo inflammatory properties. Biochemistry. 36: 2716-2723

Wuyts A, Struyf S, Gijsbers K, Schutyser E, Put W, Conings R, Lenaerts JP, Geboes K, Opdenakker G, Menten P, Proost P, Van Damme. 2003. he CXC chemokine GCP-2/CXCL6 is predominantly induced in mesenchymal cells by interleukin-1beta and is down-regulated by interferon-gamma: comparison with interleukin-8/CXCL8. J Lab Invest. 83: 23-34.

Xu D, Chan WL, Leung BP, Hunter D, Schulz K, Carter RW, McInnes IB, Robinson JH, Liew FY. 1998. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. J Exp Med. 188: 1485-1492.

Yacyshyn B, Bowen-Yacyshyn MB, Shanahan W. 1999. The clinical experience of antisense therapy to ICAM-1 in Crohn's disease. Curr Opin Mol Ther. 1: 332-335.

Yamamoto K, Takanashi S, Hasegawa Y, Kanehira Y, Kaizuka M, Okumura K. 2003. Eotaxin level in induced sputum is increased in patients with bronchial asthma and in smokers. Respiration. 70: 600-605.

Yang D, Chertov O, Bykovskaia SN. 1999. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science. 286: 525–528.

Yang J, Richmond A. 2004. The angiostatic activity of interferon-inducible protein-10/CXCL10 in human melanoma depends on binding to CXCR3 but not to glycosaminoglycan. Mol Ther. 9: 846-855.

Yoshimura T. 1987. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes. Partial characterization and separation from interleukin 1 (IL 1). J Immunol. 139: 788-93

Youn BS, Zhang S M, Broxmeyer H E, Cooper S, Antol K, Fraser M, Kwon BS. 1998. Characterization of CK8 and CK8-1: Two alternatively spliced forms of human -

hhemokine, chemoattractants for neutrophils, monocytes, and lymphocytes, and potent agonists at CC chemokine receptor 1. Blood. 91: 3118-3126

Young PR. 1998. Pharmacological modulation of cytokine action and production through signaling pathways. Cytokine and growth fac rev. 9: 239-257

Zeng X, Moore TA, Newstead MW, Deng JC, Lukacs NW, Standiford TJ. 2005. IP-10 mediates selective mononuclear cell accumulation and activation in response to intrapulmonary transgenic expression and during adenovirus-induced pulmonary inflammation. J Interferon Cytokine Res. 25: 103-112

.

Zhang L, Redington AE, Holgate ST. 1994. RANTES: a novel mediator of allergic inflammation? Clin Exp Allergy. 24: 899-904.

Zheng B, Ozen Z, Zhang X, De Silva S, Marinova E, Guo L, Wansley D, Huston DP, West MR, Han S. 2005. CXCL13 neutralization reduces the severity of collagen-induced arthritis. Arthritis Rheum. 52: 620-626.

APPENDIX I

GEArray Q Series Human Inflammatory Cytokine/Receptor Gene Array

Array Layout Table with Gene Symbol and Position Information

BLR1 1	CCR1 2	CCR2 3	CCR3 4	CCR4 5	CCR5 6	CCR6 7	CCR7 8
CCR8 9	CCR9 10	CCXCR1 11	CX3CR1 12	CXCR4 13	IFNG 14	IL10 15	IL10RA 16
IL10RB 17	IL11 18	IL11RA 19	IL12A 20	IL12B 21	IL12RB1 22	IL12RB2 23	IL13 24
IL13RA1 25	IL13RA2 26	IL15 27	IL15RA 28	IL16 29	IL17 30	IL17R 31	IL18 32
IL18R1 33	IL1A 34	IL1B 35	IL1R1 36	IL1R2 37	IL2 38	IL20 39	IL21 40
IL25 41	IL2RA 42	IL2RB 43	IL2RG 44	IL4 45	IL5 46	IL5RA 47	IL6 48
IL6R 49	IL6ST 50	IL9 51	IL9R 52	LEP 53	LTA 54	LTB 55	LTBR 56
MIF 57	SCYA1 58	SCYA11 59	SCYA13 60	SCYA14 61	SCYA15 62	SCYA16 63	SCYA17 64
SCYA18 65	SCYA19 66	SCYA2 67	SCYA20 68	SCYA21 69	SCYA22 70	SCYA23 71	SCYA24 72
SCYA25 73	SCYA3 74	SCYA4 75	SCYA5 76	SCYA7 77	SCYA8 78	SCYB10 79	SCYB11 80
SCYB13 81	SCYB5 82	SCYB6 83	SCYC1 84	SCYC2 85	SCYD1 86	SCYE1 87	SDF1 88
SDF2 89	TGFA 90	TGFB1 91	TGFB2 92	TGFB3 93	TNF 94	TNFRSF1A 95	TNFRSF1B 96
PUC18 97	PUC18 98	PUC18 99	Blank 100	Blank 101	Blank 102	GAPD 103	GAPD 104
PPIA 105	PPIA 106	PPIA 107	PPIA 108	RPL13A 109	RPL13A 110	ACTB 111	ACTB 112

Gene List

Position	UniGene	Genebank	Symbol	Description	Gene name
1	Hs.113916	NM_001716	BLR1	Homo sapiens Burkitt lymphoma receptor 1, GTP-binding protein (BLR1)	CXCR5 (BLR1)
2	Hs.301921	NM_001295	CCR1	Chemokine (C-C motif) receptor 1	CCR1
3	Hs.395	NM_000648	CCR2	Chemokine (C-C motif) receptor 2	CCR2
4	Hs.158324	NM_001837	CCR3	Chemokine (C-C motif) receptor 3	CCR3
5	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4	CCR4
6	Hs.54443	NM_000579	CCR5	Chemokine (C-C motif) receptor 5	CCR5
7	Hs.46468	NM_004367	CCR6	Chemokine (C-C motif) receptor 6	CCR6
8	Hs.1652	NM_001838	CCR7	Chemokine (C-C motif) receptor 7	CCR7
9	Hs.113222	NM_005201	CCR8	Chemokine (C-C motif) receptor 8	CCR8
10	Hs.225946	NM_006641	CCR9	Chemokine (C-C motif) receptor 9	CCR9
11	Hs.248116	NM_005283	CCXCR1	Homo sapiens chemokine (C motif) XC receptor 1 (CCXCR1)	XCR1
12	Hs.78913	NM_001337	CX3CR1	Chemokine (C-X3-C) receptor 1	CX3CR1
13	Hs.89414	NM_003467	CXCR4	Chemokine (C-X-C motif), receptor 4 (fusin)	CXCR4
14	Hs.856	X13274	IFNG	Interferon, gamma	IFN-r
15	Hs.193717	M57627	IL10	Interleukin 10	IL-10
16	Hs.327	NM_001558	IL10RA	Interleukin 10 receptor, alpha	IL-10Ra
17	Hs.173936	Z17227	IL10RB	Interleukin 10 receptor, beta	IL-10Rb
18	Hs.1721	M57765	IL11	Interleukin 11	IL-11
19	Hs.64310	NM_004512	IL11RA	Interleukin 11 receptor, alpha	IL-11Ra
20	Hs.673	M65271	IL12A	Interleukin 12A, p35	IL-12A
21	Hs.674	M65272	IL12B	Interleukin 12B, p40	IL-12B
22	Hs.121544	NM_005535	IL12RB1	Interleukin 12 receptor, beta 1	IL-12Rb1
23	Hs.73165	NM_001559	IL12RB2	Interleukin 12 receptor, beta 2	IL-12Rb2
24	Hs.845	NM_002188	IL13	Interleukin 13	IL-13
25	Hs.285115	NM_001560	IL13RA1	Interleukin 13 receptor, alpha 1	IL13RA1
26	Hs.25954	Y08768	IL13RA2	Interleukin 13 receptor, alpha 2	IL-13Ra2
27	Hs.168132	AF031167	IL15	Interleukin 15	IL-15
28	Hs.12503	NM_002189	IL15RA	Interleukin 15 receptor, alpha	IL-15Ra
29	Hs.82127	M90391	IL16	Interleukin 16 (lymphocyte chemoattractant factor)	IL-16
30	Hs.41724	U32659	IL17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	IL-17
31	Hs.129751	U58917	IL17R	Homo sapiens IL-17 receptor mRNA	IL-17 R
32	Hs.63077	NM_001562	IL18	Interleukin 18 (interferon-gamma-inducing factor)	IL-18
33	Hs.159301	NM_003855	IL18R1	Interleukin 18 receptor 1	IL18R1
34	Hs.1722	M28983	IL1A	Interleukin 1, alpha	IL-1a
35	Hs.126256	M15330	IL1B	Interleukin 1, beta	IL-1b
36	Hs.82112	NM_000877	IL1R1	Interleukin-1 receptor type I	IL-1R1
37	Hs.25333	U74649	IL1R2	Interleukin-1 receptor type II	IL-1R2
38	Hs.89679	U25676	IL2	Interleukin 2	IL-2
39	Hs.272373	NM_018724	IL20	Interleukin 20	IL20
40	Hs.302014	NM_021803	IL21	Homo sapiens interleukin 21 (IL21)	IL21
41	Hs.10927	AL365373	IL25	Likely ortholog of mouse interleukin 25	IL25
42	Hs.1724	X01057	IL2RA	Interleukin 2 receptor, alpha	IL-2 Ra
43	Hs.75596	NM_000878	IL2RB	Interleukin 2 receptor, beta	IL-2 Rb
44	Hs.84	NM_000206	IL2RG	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	IL-2 Rr
45	Hs.73917	M13982	IL4	Interleukin 4	IL-4
46	Hs.2247	X04688	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	IL-5
47	Hs.68876	M75914	IL5RA	Interleukin 5 receptor, alpha	IL-5 Ra

Position	UniGene	Genebank	Symbol	Description	Gene name
48	Hs.93913	M14584	IL6	Interleukin 6 (interferon, beta 2)	IL-6
49	Hs.193400	NM_000565	IL6R	Interleukin 6 receptor	IL-6 Ra
50	Hs.82085	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	gp130
51	Hs.960	X17543	IL9	Interleukin 9	IL-9/p40
52	Hs.1702	NM_002186	IL9R	Interleukin 9 receptor	IL-9 Ra
53	Hs.194236	NM_000230	LEP	Leptin (murine obesity homolog)	Leptin
54	Hs.36	D12614	LTA	Lymphotoxin-alpha (TNF superfamily, member 1)	TNF-b/Lta
55	Hs.890	NM_002341	LTB	Lymphotoxine-beta	LT-b
56	Hs.1116	L04270	LTBR	Homo sapiens lymphotoxin beta receptor (TNFR superfamily, member 3(LTBR)	LTbR
57	Hs.73798	NM_002415	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF
58	Hs.72918	M57502	SCYA1	Small inducible cytokine A1 (I-309, homologous to mouse Tca-3)	I-309
59	Hs.54460	NM_002986	SCYA11	Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)	Eotaxin
60	Hs.11383	NM_005408	SCYA13	Small inducible cytokine subfamily A (Cys-Cys), member 13	MCP-4
61	Hs.20144	Z49270	SCYA14	Small inducible cytokine subfamily A (Cys-Cys), member 14	HCC-1
62	Hs.272493	NM_004167	SCYA15	Small inducible cytokine subfamily A (Cys-Cys), member 15	MIP-1 delta
63	Hs.10458	U91748	SCYA16	Small inducible cytokine subfamily A (Cys-Cys), member 16	HCC-4
64	Hs.66742	NM_002987	SCYA17	Small inducible cytokine subfamily A (Cys-Cys), member 17	TARC (SCYA17)
65	Hs.16530	AB000221	SCYA18	Small inducible cytokine subfamily A (Cys-Cys), member 18, pulmonary and activation-regulated	PARC
66	Hs.50002	NM_006274	SCYA19	Small inducible cytokine subfamily A (Cys-Cys), member 19	SCYA19
67	Hs.303649	X14768	SCYA2	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-e)	MCP-1(SCYA2)
68	Hs.75498	U77035	SCYA20	Small inducible cytokine subfamily A (Cys-Cys), member 20	MIP-3a
69	Hs.57807	NM_002989	SCYA21	Small inducible cytokine subfamily A (Cys-Cys), member 21	MIP-2 (SCYA21)
70	Hs.97203	NM_002990	SCYA22	Small inducible cytokine subfamily A (Cys-Cys), member 22	MDC
71	Hs.169191	U85767	SCYA23	Small inducible cytokine subfamily A (Cys-Cys), member 23	MPIF-1
72	Hs.247838	NM_002991	SCYA24	Small inducible cytokine subfamily A (Cys-Cys), member 24	MPIF-2
73	Hs.50404	NM_005624	SCYA25	Human chemokine (TECK)	TECK
74	Hs.73817	M23452	SCYA3	Small inducible cytokine A3 (homologous to mouse Mip-1a)	MIP-1a
75	Hs.75703	NM_002984	SCYA4	Small inducible cytokine A4 (homologous to mouse Mip-1b)	MIP-1b
76	Hs.241392	NM_002985	SCYA5	Small inducible cytokine A5 (RANTES)	SCYA5 (RANTES)
77	Hs.251526	X72308	SCYA7	Homo sapiens mRNA for monocyte chemotactic protein-3 (MCP-3)	MCP-3
78	Hs.271387	Y10802	SCYA8	Small inducible cytokine subfamily A (Cys-Cys), member 8 (monocyte chemotactic protein 2)	MCP-2

Position	UniGene	Genebank	Symbol	Description	Gene name
79	Hs.2248	NM_001565	SCYB10	Gamma-interferon inducible early response gene (small inducible cytokine subfamily B (Cys-X-Cys)	IP10 (IP 10)
80	Hs.103882	Y15220	SCYB11	Small inducible cytokine subfamily B (Cys-X-Cys), member 11	I-TAC (IP9) (SCYB11)
81	Hs.100431	NM_006419	SCYB13	Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	SCYB13
82	Hs.89714	NM_002994	SCYB5	Small inducible cytokine subfamily B (Cys-X-Cys), member 5 (epithelial-derived neutrophil-activating	ENA-78
83	Hs.184021	NM_002993	SCYB6	Human chemokine alpha 3 (CKA-3) mRNA	GCP-2
84	Hs.3195	U23772	SCYC1	Small inducible cytokine subfamily C, member 1 (lymphotactin)	lymphotactin
85	Hs.174228	D63789	SCYC2	Small inducible cytokine subfamily C, member 2	SCYC2
86	Hs.80420	U91835	SCYD1	Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin)	Fractalkine
87	Hs.333513	NM_004757	SCYE1	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	SCYE1
88	Hs.237356	U16752	SDF1	Stromal cell-derived factor 1	SDF1
89	Hs.118684	D50645	SDF2	Homo sapiens mRNA for SDF2	SDF2
90	Hs.170009	NM_003236	TGFA	Transforming growth factor, alpha	TGF-a
91	Hs.1103	X02812	TGFB1	Transforming growth factor, beta 1	TGFb1
92	Hs.169300	M19154	TGFB2	Transforming growth factor, beta 2	TGF b2
93	Hs.2025	NM_003239	TGFB3	Transforming growth factor, beta 3	TGF b3
94	Hs.241570	X01394	TNF	Tumor necrosis factor (TNF superfamily, member 2)	TNFa
95	Hs.159	M33294	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	TNFR1
96	Hs.256278	NM_001066	TNFRSF1B	Human tumor necrosis factor receptor 2	TNFR2 (TNFSF1B)
97	N/A	L06752	PUC18	PUC18 Plasmid DNA	pUC18
98	N/A	L06752	PUC18	PUC18 Plasmid DNA	pUC18
99	N/A	L06752	PUC18	PUC18 Plasmid DNA	pUC18
100	Blank	Blank	Blank	Blank	0
101	Blank	Blank	Blank	Blank	0
102	Blank	Blank	Blank	Blank	0
103	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
104	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
105	Hs.182937	NM-021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	cyclophilin A
106	Hs.182937	NM-021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	cyclophilin A
107	Hs.182937	NM-021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	cyclophilin A
108	Hs.182937	NM-021130	PPIA	Homo sapiens peptidylprolyl isomerase A (cyclophilin A) (PPIA)	cyclophilin A
109	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
110	Hs.119122	NM_012423	RPL13A	Ribosomal protein L13a (23 Kda highly basic protein)	RPL13A
111	Hs.288085	X00355	ACTB	Beta Actin	b-actin
112	Hs.288085	X00355	ACTB	Beta Actin	b-actin